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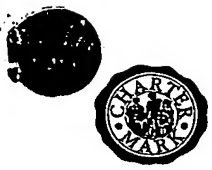

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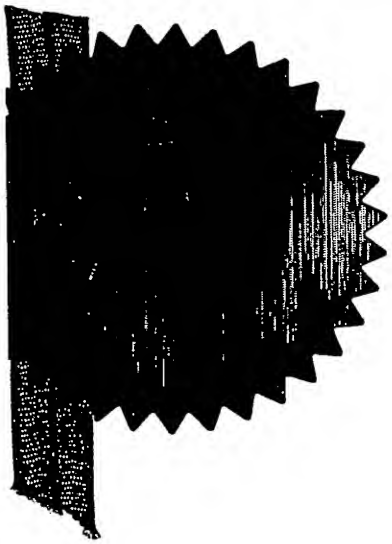

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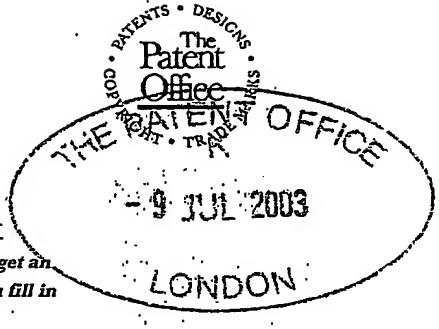

10JUL03 E821405-1 D00001
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1. Your reference 9452 GB/CAH

2. Patent application number *(The Patent Office will fill in this part)* 0316075.1 09 JUL 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
   Molecular Sensing plc
   3 Challeymead Business Park
   Bradford Road
   Melksham
   Wiltshire SN12 8LH

   Patents ADP number *(if you know it)* 7836813001

   If the applicant is a corporate body, give the country/state of its incorporation United Kingdom

4. Title of the invention
   PROTEASE DETECTION ASSAY

5. Name of your agent *(if you have one)* Abel & Imray

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*
   20 Red Lion Street
   London
   WC1R 4PQ
   United Kingdom

   Patents ADP number *(if you know it)* 174001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month /*

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Signature Abel + Imray Date 9 July 2003

Abel & Imray

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PROTEASE DETECTION ASSAY

FIELD OF THE INVENTION

The invention relates to methods for the detection or assessment of protease activity, and to substrates and apparatus for use in such methods.

BACKGROUND OF THE INVENTION

Protease enzymes are involved in a variety of biological phenomena, for example, in protein activation and cell signalling. Protease activity plays a key role in processes such as blood clotting, apoptosis and hormone regulation. Proteases are also essential to the function of a variety of viral and microbial pathogens. There is an increasing interest in the development of protease inhibitors for use as therapeutic agents.

The determination of protease activity in biological samples is important in the analysis of processes such as apoptosis, in the screening of potential protease inhibitors and in the monitoring of sample purity, for example during protein purification. Protease enzymes hydrolyse amides and esters to produce peptides, single amino acids or labelled amino acid fragments depending on the structure of the substrate and the nature of the enzyme. The determination of protease activity is most commonly performed using either a naturally occurring protein substrate or a synthetic peptide substrate analogue that is labelled, for example with a fluorophore or a chromophore.

The nature of the protein used depends on the solubility required and the particular requirements of the assay; bovine serum albumin (BSA) or casein are commonly used. Gelatin, ovalbumin and cross-linked proteins have also been used.

Most detection assays on the market use a labelled substrate analogue. The detection of protease activity can be performed using a homogenous or a heterogenous reaction set up. In a homogenous detection assay, the substrate is typically in solution and the product is also in solution. Fluorescence-based detection systems using fluorophores (for example fluoroscein, rhodamine or BODIPY fluorophores) generally operate according to one of two principles: detection of a fluorescent signal following cleavage of a multiply labelled self-quenched protein (enzChek protease assay kits, Molecular Probes Inc.), or detection of a change in the size of the fluorescent-labelled moiety/conjugate using fluorescence polarization techniques (Beacon ®, protease activity detection kit, PanVera Corporation, enzCheck polarization kit, Molecular Probes Inc.).

In one commonly practised method, a peptide substrate is used that is labelled at the carboxy terminus with a dye possessing an amine functionality. Such a dye may be a chromophore or a fluorophore, for example coumarin, fluoroscein, rhodamine or BODIPY (Molecular Probes Inc, apoalert™ , CPP32 protease assay kits, Clontech). The amide bond that couples dye and amino acid is cleaved by the protease to produce an amine derivative. That change in structure affects the spectral characteristics of the dye and a detectable signal is thus produced. An alternative strategy for homogenous detection is to use a peptide substrate that is labelled at one side of a cleavage site with a donor fluorophore and at the other side with an acceptor quencher, which together form a fluorescence resonance energy transfer (FRET) pair. Cleavage of the peptide results in separation of the donor and acceptor and therefore produces a change in fluorescence signal.

Hydrolysis of a specific peptide sequence can be detected by a gel based analysis (Peptag protease assay, Promega).

A heterogenous assay typically involves the cleavage of a dye-labelled fragment of an immobilised substrate and subsequent analysis of the liquid phase (ProteaseSpots, Jerini, ProCheck™ Universal Protease Assay, Intergen).

Protease activity may also be determined using an unmodified (i.e. naturally occurring) protein substrate. Assays using unmodified protein substrates generally require precipitation of the undigested substrate and subsequent detection of cleaved protein fragments. Such detection can be, for example, by measurement of absorbance at 278 nm, or by detection of the resulting primary amine functionality. Such methods often lack sensitivity, require sampling to obtain kinetic data and depend on quantitative precipitation for accurate results. Alternatively, hydrolysis of succinylated proteins may be detected following reaction of the resulting peptide fragments with TNBSA (trinitrobenzenesulfonic acid).

Except where the contrary is apparent from the context, the term "substrate" is used throughout the remainder of this document to include both naturally occurring substrates and synthetic substrate analogues.

The term "protease" as used herein is intended to include within its scope proteins that are known as proteinases.

The term "peptide" and "protein" are used interchangeably herein and both include amino acid sequences of any length including those with a small number of amino acid residues, for example five residues. The terms "peptide" and "protein" include both molecules made in cells and molecules made by cell-free synthesis. The terms "peptide" and "protein" include molecules having naturally

occurring, semi-synthetic or artificial sequences, which sequences may include amino acids that do not occur naturally in proteins. For example, the terms "peptide" and "protein" refer to an amino acid sequence of a recombinant or non-recombinant peptide having an amino acid sequence of (1) a native peptide, (ii) a biologically active fragment of a native peptide, (iii) a biologically active peptide analogue of a native peptide, or (iv) a biologically active variant of a native peptide.

SUMMARY OF THE INVENTION

The invention provides a method of detecting protease activity in a sample comprising contacting a sample solution with a protease substrate labelled with an electrochemically active marker, providing conditions under which any protease which may be present in the sample may degrade the protease substrate, and electrochemically determining information relating to the electrochemically active marker. The information relating to the marker is expediently used to derive information concerning the presence or absence of protease activity. Preferably the electrochemical information may be used to quantify relative proportions of degraded and non-degraded substrate. As used herein the term "degrade" includes any degradation as a result of enzyme activity, for example by digestion.

The modification of various biological molecules with a redox active label is known. Ferrocene is a commonly used label for such purposes because of its stability and its electrochemical properties and because of the availability of suitable ferrocene derivatives. For example a ferrocene modified glucose oxidase in which ferrocene is covalently attached to the surface of glucose oxidase via a spacer molecule was synthesised and that modified enzyme was found

to allow for more efficient electron transfer between the enzyme and the mediator (International Journal of Biological Macromolecules, 1992, **14(4)**, 210-214). Ferrocene has also been used to label proteins so as to facilitate their detection by voltammetric methods (for example BSA, avidin or cytochrome P450). Ferrocenylated biological molecules for conjugation with a second molecule are also known, for example ferrocene-labelled digoxin antibody, ferrocene-labelled anti-HCG IgG and ferrocene-labelled biotin.

Modification of proteins with ferrocene may be performed for example by covalent attachment of an amine-, carboxyl- or sulfhydryl-reactive ferrocene derivative with specific amino acid residues of the protein. The examples of ferrocene derivatives developed to date include diamides, succinimidyl esters, aldehydes, primary amines, iodoacetamides and maleimides.

DETAILED DESCRIPTION

The application of electrochemical detection to protease assays has a number of advantages over fluorescent detection. Electrochemical detection has the potential for very high levels of sensitivity and exhibits a wider linear dynamic range than fluorescence. There is no requirement for samples to be optically clear. There is also less interference from background contaminants (many biological samples auto fluoresce).

The present invention is based on the observation that an electrochemically active marker exhibits different electrochemical characteristics depending on whether or not it is attached to an amino acid residue, on whether or not that amino acid residue is incorporated into a peptide or protein, and on the length of any such peptide or protein.

The size and characteristics of a molecule to which an electrochemically active marker is attached influence the observable characteristics of the electrochemical marker. That may occur, for example, by influencing the rate of migration of the marker by diffusion or its rate of migration in response to an electric field.

Electrochemical activity of a marker may also be influenced by steric effects resulting from the presence of the molecule to which it is linked. For example, steric hindrance may prevent the marker from approaching an electrode and accepting or donating electrons.

If the marker is attached to a peptide then the secondary structure of the peptide (as largely determined by the primary sequence) may influence the physical properties of the marker. For example, if the marker is attached to an amino acid residue in a peptide such that the structure of the peptide sterically hinders the electrochemically active marker then the signals observable by voltammetry may be reduced. Digestion of the peptide may destroy or release secondary structure elements and thus reduce or abolish the influence of the peptide structure on the marker. Accordingly, digestion of the peptide results in a change, usually an increase, in the electrochemical signal produced by the marker moiety. In a differential pulse voltammetry experiment, the faradaic current response at a particular applied voltage may increase upon digestion of the peptide.

It will be understood by the person skilled in the art that, because the secondary structure of a peptide is dependent on temperature, the effects the peptide has on an electrochemically active marker varies with temperature. The person skilled in the art is able to select a temperature at which to carry out the electrochemical technique of the invention in order to achieve an optimal signal to background

noise ratio for the technique. If the technique is incorporated into an assay in which heating or cooling is performed, a measurement at a desired temperature can simply be obtained by selecting an appropriate point in the temperature regime to make the measurement.

The information relating to the electrochemically active marker can be obtained by voltammetry or by an amperometric method. Differential pulse voltammetry is particularly suitable. If desired, the electrochemical detection step may be carried out using one or more electrodes covered by a membrane which is able selectively to exclude molecules based on one or more characteristics, for example size, charge or hydrophobicity. That may assist in eliminating background noise current arising from, for example, charged species in the solution.

Suitable electrochemically active markers include those comprising metallo-carbocyclic pi complexes, that is organic complexes with partially or fully delocalised pi electrons. Suitable markers include those comprising sandwich moieties, in which two carbocyclic rings are parallel, or bent sandwich compounds (angular compounds) and monocyclopentadienyls. Preferably the electrochemically active marker is a metallocenyl label. More preferably it is a ferrocenyl label.

The ferrocene or metallocene ring, which constitutes the labelling moiety, may be unsubstituted. If desired, the ferrocene or metallocene ring structure may be substituted by one or more substituents, the nature and location of which are selected so as to influence in a desired manner the redox characteristics of the ferrocene or metallocene moiety. The ferrocene or metallocene ring may additionally or instead be substituted by any ring substituents that do not materially

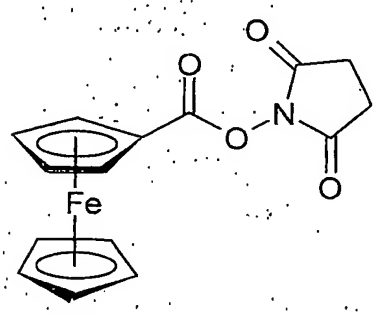
reduce the electrochemical sensitivity of the label. Ferrocenyl and metallocenyl markers may advantageously be *N*-substituted ferrocene or metallocene carboxamides. The ferrocene or metallocene carboxamide moiety may be linked via the carboxamide nitrogen to the protein or peptide. Linkage to the protein or peptide can be by any suitable linkage, typically by linkage to an amino acid side chain, where linkage is via an amine group, the nitrogen atom may constitute the nitrogen of the carboxamide moiety. Various synthetic methods have been developed for the derivatisation of protein or peptide amino acid side chains or protein or peptide terminal amino acids. For example, lysine residues in a protein may be derivatised by reaction with a succinimidyl ester. For derivatisation at other amino acid residues, other known synthetic methods may be used. For example, a maleimide reagent may be used to derivatise cysteine residues. An N-hydroxy succinimide ester may be used to label the amino terminus of protein or peptide.

The marker group may be attached to the peptide or protein through a linker moiety. There may be used any suitable linker moiety. Suitable linker moieties may comprise an aliphatic chain which may be linear or branched, and saturated or unsaturated. Advantageously, the linker moiety is a linear or branched aliphatic chain having from 4 to 20 carbon atoms, and preferably from 6 to 16, especially from 8 to 14 atoms, especially 12 carbon atoms. The alkylene chains may be substituted by any substituent or may be interrupted by any atom or moiety provided that any such substituent, atom or moiety does not materially reduce the electrochemical sensitivity of the marker.

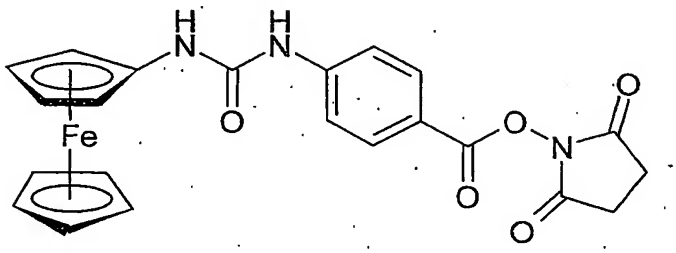
Illustrative of the ferrocenyl labels which may be used in accordance with the invention are those in Formulae I and

II. In formulae Ia and IIa, the same labels are shown attached to a lysine amino acid residue of a peptide.

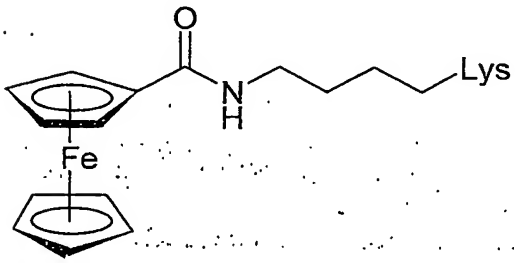
Formula I



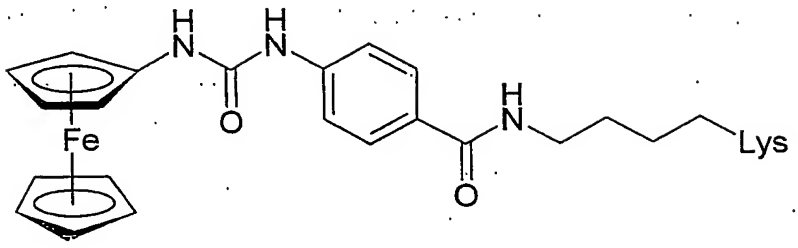
Formula II



Formula Ia



Formula IIa



In the case of a peptide or protein with significant secondary structure, the synthesis of a labelled protein or peptide starting from a labelling moiety and the appropriate protein or peptide does not generally result in the complete derivatisation of the protein or peptide. Many sites are not accessible to reagents in solution. For example, the BSA molecule comprises 60 lysine residues (Hirayama *et al.*, *Biochem. Biophys. Res. Commun.*, 1990, **173**, 639-646). According to the literature, labelling of BSA with succinimidyl esters has given BSA:label ratios of between 1:5 and 1:23 (Jones, L.J. *et al.*, *Analytical Biochem.*, 1997, **251**, 144-152 and Hiroaki, S. *et al.*, *Sensors and Actuators B*, 2000, **65**, 144-146.). Generally the reaction product of a labelling synthesis contains a mixture of different product molecules with different numbers of markers. The average number of labels can be assessed by various spectroscopic methods, for example UV-visible spectroscopy. The distribution of the numbers of markers per protein molecule can be assessed more precisely by use of mass spectrometry.

The precise number of marker moieties present on a protein or peptide is not critical to the success of the assay of the invention. For good sensitivity, there are

preferably on average several marker moieties on each protein or peptide molecule. For use in assays with enzymes that cleave only particular amino acid sequences, it is preferable that a marker moiety is located relatively close to the cleavage site such that the marker's immediate environment is affected by the cleavage and its observable electrochemical properties are affected.

Many protease substrate molecules are proteins comprising several hundred amino acid residues. Whilst use of a substrate analogue that is a full length peptide (that is, is of length the same as or similar to that of a substrate for which it is an analogue) may be useful in some circumstances. For example, an analogue which is of similar length to a naturally occurring substrate for which it is an analogue might be expected to have characteristics (such as stability and conformational characteristics) which are more similar to those of the natural substrate than would be those of a much shorter analogue, and might therefore more accurately mimic the behaviour of the natural substrate. In many circumstances, however, it is not essential for a full length protein to be used. A minimum of 5 amino acid residues in a peptide is generally required. Preferably, the peptide comprises at least 20 amino acid residues. For example, the peptide may comprise from 20 to 100 amino acid residues; most preferably the peptide comprises from 20 to 50 amino acid residues. In practice, the length of the peptide is so selected that there is present at least one cleavage site for the enzyme or enzymes of interest. Preferably the peptide has one cleavage site for the enzyme or enzymes of interest. Peptides having more than one cleavage site may be of use, for example, where the substrate is to be used in a screen for general protease activity.

The method of the invention may be used to offer a qualitative measure of the protease activity in an unknown sample. The amount of protease activity may be quantified, for example, with use of a calibration curve obtained with standard solutions. If the identity of the protease present in a sample is known, the concentration of the protease may be calculated.

The invention further provides a protease assay kit comprising a protease substrate labelled with an electrochemically active marker. Such a kit may also comprise further reagents, for example appropriate solutions. The kit may also comprise instructions for carrying out a protease determination.

The invention also provides a novel alternative electrochemically active labelled protein or peptide. The invention accordingly provides a compound of formula III,

$$Mc\text{-}NR'\text{-}C(=O)\text{-}X\text{-}(Ar)_n\text{-}(L)_m\text{-}R \qquad \text{III}$$

wherein

- Mc is a metallocenyl group in which each ring may independently be substituted or unsubstituted,
- the metallocenyl group comprises a metal ion M selected from the group consisting of iron, chromium, cobalt, osmium, ruthenium, nickel or titanium,
- R′ is H or lower alkyl,
- X is either NR′ or O,
- Ar is a substituted or unsubstituted aryl group,
- n is 0 or 1,
- L is a linker group,
- m is 0 or 1, and
- R is a protein or peptide.

The Mc group may be substituted by one or more groups selected from lower alkyl (for example C_1 to C_4 alkyl); lower alkyl substituted with a hydroxy, halo, cyano, oxo, amino, ester or amido or a further metallocene group; lower alkenyl; lower alkenyl substituted with a hydroxy, halo, cyano, oxo, amino, ester or amido, or a further metallocene group; aryl;or , aryl substituted with a hydroxy, halo, cyano, oxo, amino, ester or amido, or a further metallocene group. The further metallocene group, if present, may be substituted in the same way as the Mc group with the exception that the total number Mc groups in the molecule of the invention preferably does not exceed four. Preferably, the Mc group is unsubstituted.

Preferably, M is an ion selected from iron, osmium or ruthenium. Most preferably, M is an iron ion. When M is an iron ion, Mc is a ferrocene.

Lower alkyl is preferably C1 to C4 alkyl. Preferably, R′ is H. Each R′ has an identity separate from the other R′.

Preferably X is NH.

The Ar group preferably has at least 5, and for example from 5 to 10, ring carbon atoms, with C_6-aryl being preferred. The Ar group may be substituted by one or more groups selected from lower alkyl (for example C_1 to C_4 alkyl); lower alkyl substituted with a hydroxy, halo, cyano, oxo, amino, ester or amido group; lower alkenyl; lower alkenyl substituted with a hydroxy, halo, cyano, oxo, amino, ester or amido group; aryl; and aryl substituted with a hydroxy, halo, cyano, oxo, amino, ester or amido group. Preferably, the Ar group is unsubstituted.

Preferably, n=1. Preferably, m=1. In one preferred embodiment, n=1 and m=1.

Suitable linker groups L include any moiety that is suitable for linking an amino group of a protein to an adjacent Ar or X group, respectively, and the selection of such groups would be a matter of routine for those skilled in the art. By way of example, the linker group L may be carbonyl, or may be an aliphatic chain which may be linear or branched, and saturated or unsaturated. Advantageously, the linker moiety is a linear or branched aliphatic chain having from 1 to 20 carbon atoms, preferably with at least 4 carbon atoms, and more preferably from 6 to 16, especially from 8 to 14 atoms, more especially 12 carbon atoms. The linker moiety may be an alkylene chain which may be substituted by any substituent or may be interrupted by any atom or moiety provided that any such substituent, atom or moiety does not materially reduce the electrochemical sensitivity of the label.

Preferably, R is a protein or a peptide of more than 10 amino acid residues in length, more preferably more than 20 amino acid residues in length, still more preferably more than 40 amino acid residues in length. Preferably, R is a protein that is a substrate for a protease enzyme. In a preferred embodiment, R is BSA, or casein. Other suitable protease substrates may include gelatine, elastin, collagen and ovalbumin, fluorescently labelled forms of each of which are available from Molecular Probes, Inc.

In one class of labelled protein in accordance with the invention, the marker moiety is attached to an amino group which is contained within a pendant side chain of the

protein, and is preferably at the free amino terminus of that pendant side chain. That side chain may be a side chain which is part of a naturally occurring amino acid in which it is present. Thus, in Formula IIa above, the $-NH-(CH_2)_4-$chain is derived from the $NH_2(CH_2)_4-$chain which is inherently present in lysine. It will be appreciated, however, that a suitable pendant side chain may if desired be synthetically introduced into any desired location in the protein.

In one preferred class of labelled proteins, illustrated by formula IIa above:

Mc is ferrocenyl, R' is H, X is -NH-, Ar is phenylene, L is carbonyl and n=m=1, and R presents a protein in which the marker moiety is attached to the protein backbone via a pendant side chain of a lysine residue.

In another class of labelled protein in accordance with the invention, the marker moiety may be attached via the terminal amino group of the protein, and in that case it will normally be preferred for L to have a minimum of four carbon atoms, preferably comprising a carbonyl group which in the labelled protein combines with the protein terminal amine group to form a carboxamide moiety.

In a yet further class of labelled protein in accordance with the invention, the marker moiety is attached via a pendant side chain which has been synthetically attached to the protein backbone and in that case L will normally comprise a chain of minimum of four carbon atoms having at one end of said chain, or being interrupted by, a carboxamide moiety.

The compound of the invention may comprise more than one metallocene groups. Typically, several metallocene groups are attached to the same protein or peptide molecule. For

example, in the case of BSA, there may be 10 to 20 metallocene groups per BSA molecule. In the compound of the invention, the metallocene group may be substituted by any other electrochemically active marker group. The compound of the invention may be one which is electrochemically active or becomes electrochemically active following partial cleavage.

Compounds of the invention may be prepared by reaction of a metallocene compound comprising a suitable functional group with a protein or peptide

For example, an *N*-hydroxysuccinimide ester of a metallocene derivative may be used. Details of the use of such a compound to label a peptide are provided in Examples 2, 3 and 4. *N*-hydroxysuccinimide esters are suitable for attachment of a marker to lysine side chains. It will however be apparent to the skilled person that similar labels may be attached to a peptide at any suitable side chain by use of an appropriate labelling functional group.

Compounds in accordance with the invention have particular utility in methods according to the invention. Under the conditions set out in table 1, substituted ferrocene carboxylic acids have an electrode potential in the region of 400mV. On the other hand, substituted metallocene compounds in accordance with the invention have an electrode potential in the region of 150mV. The lower potential is a potential at which the propensity for background impurities to interfere with data collection is much lower. Accordingly, the compounds of the invention enable more sensitive readings to be taken. In figure 12 there are shown voltammograms of digestion of a 4-(3'-ferrocenylureido)-1-benzoyl labelled BSA molecule; in figure 3, there are shown voltammograms of digestion of a ferrocenyl labelled BSA molecule under the same reaction conditions. As is seen from a comparison of figures 12(c) and 3(c) the peak for the 4-

(3'-ferrocenylureido)-1-benzoyl derivative with a ferrocene moiety as found in molecules of the invention comes at around 100mV, whereas the peak for the ferrocenyl derivative comes at around 400mV.

The invention also provides apparatus arranged to carry out any one or more of the methods disclosed herein. Such apparatus may include suitable electrodes, electrochemical cells, disposable plastic ware and apparatus for detecting, recording, manipulating and displaying results. A thermostat device may also be included.

The invention provides apparatus comprising one or more sample receiving regions for receiving one or more samples, means for controlling the temperature of said sample receiving regions and means for measuring the electrochemical properties of said sample. Such an apparatus may be manufactured so as to utilize conventional electrode cells (for example those used in examples herein).

The present invention further provides a container comprising one or more sample receiving regions for holding one or more samples. Such a container may be based on the design of polypropylene tubes or 96-well plates as presently used in many molecular biological applications. Ideally such a container will be adapted to receive at least one electrode component. That electrode component might, for example, be located as part of a lid for the container so that when it is used to close the container, the electrode component(s) reach into the sample solution. Conventional electrochemical cells are generally not regarded as disposable because of their relatively high cost. The use of disposable plastic ware has become standard practice in molecular biology because it mitigates the risks of sample contamination.

Certain illustrative embodiments of the invention will now be described in detail with reference to the accompanying drawings in which:

Fig. 1 is a schematic representation of an electrochemical cell used in differential pulse voltammetry measurements described herein;

Fig. 2 is a set of overlaid UV-visible spectra for ferrocene and BSA conjugates and mixtures;

Figs. 3a, 3b and 3c are differential pulse voltammograms of trypsin digestion products of Fc-BSA as described in Example 5A;

Figs. 4a, 4b and 4c are differential pulse voltammograms of α-chymotrypsin digestion products of Fc-BSA as described in Example 5B;

Figs. 5a, 5b and 5c are differential pulse voltammograms of elastase digestion products of Fc-BSA as described in Example 5C;

Figs. 6a, 6b and 6c are differential pulse voltammograms of pepsin digestion products of Fc-BSA as described in Example 5D;

Figs. 7a, 7b and 7c are differential pulse voltammograms of carboxypeptidase digestion products of Fc-BSA as described in Example 5E;

Figs. 8a, 8b and 8c are differential pulse voltammograms of thermolysin digestion products (at 37°C) of Fc-BSA as described in Example 5F;

Figs. 9a, 9b and 9c are differential pulse voltammograms of thermolysin digestion products (at 70°C) of Fc-BSA as described in Example 5G;

Figs. 10a and 10b are differential pulse voltammograms of trypsin digestion products of unlabelled BSA as described in Example 5H;

Figs. 11a, 11b and 11c are differential pulse voltammograms of trypsin digestion products of FcU-BSA as described in Example 5I;

Figs. 12a, 12b and 12c are differential pulse voltammograms of papain digestion products of FcU-BSA as described in Example 5J;

Figs. 13a, 13b and 13c are differential pulse voltammograms of trypsin digestion products of FcU-casein as described in Example 5K;

Fig. 14 shows overlaid differential pulse voltammograms of products of trypsin digestion of Fc-BSA carried out at various concentrations of enzyme as described in Example 6;

Fig. 15 shows overlaid differential pulse voltammograms of products of trypsin digestion of Fc-BSA carried out with various incubation times as described in Example 7;

Fig. 16 shows overlaid differential pulse voltammograms of products of trypsin digestion of Fc-BSA carried out with various concentrations of protease inhibitor present as described in Example 8;

Fig. 17 shows overlaid amperometric traces of trypsin digestion of Fc-BSA with time;

Fig. 18 shows overlaid amperometric traces of papain digestion of FcU-BSA with time;

With reference to Fig. 1, an electrochemical cell 1 suitable for use in the cyclic voltammetry experiments described herein comprises a vessel 2, containing a background electrolyte solution 3, which is an aqueous 100mM solution of sodium chloride. Immersed in the solution 3 is a chamber 4, in which is located the sample to be tested and, immersed therein, a glassy carbon working electrode 5. A gold electrode may alternatively be used. Also immersed in the solution 3 is a counter-electrode 6 of platinum wire and

a silver/silver chloride reference electrode 7 immersed in 4M potassium chloride solution, which solutions are in communication with others via a sintered disc.

The following Examples illustrate the invention:

MATERIALS AND METHODS – ferrocenylated BSA preparations and assays

Bovine serum albumin (lyophilised powder, approx. 99%), casein (bovine milk, purified powder), porcine pancreas trypsin (1120 BAEE units/mg solid), type II bovine pancreas α-chymotrypsin (51 units/mg solid), porcine stomach mucosa pepsin (632 units/mg solid), *Bacillus thermoproteolyticus rokko* thermolysin (44 units/mg solid), Tritirachium album proteinase K (33 units/mg solid), papaya latex papain (14 units/mg protein, 99%) and type I-S soybean trypsin inhibitor were obtained from Sigma.

Ferrocene carboxylic acid was obtained from Aldrich Chemical Co.

Potassium bicarbonate (A.C.S. reagent), potassium carbonate (minimum 99%), and dimethyl sulfoxide (ACS reagent, min. 99.9%) were obtained from Sigma.

NAP 10 columns (G25 DNA grade sephadex) were obtained from Amersham Biosciences. Trizma hydrochloride (99+%), Trizma base (99+%), sodium chloride (SigmaUltra min. 99.5%), sodium acetate (molecular biology grade), ethylenediaminetetraacetic acid tetrasodium salt (SigmaUltra min. 99.0%), sodium hydroxide (SigmaUltra min. 98%), DL-cystein hydrochloride (min. 98%), hydrochloric acid, and molecular biology grade

water were obtained from Sigma. Ponceau S (practical grade) ammonium persulfate (electrophoresis reagent), N,N,N',N'-tetramethyleneethylenediamine (TEMED), acrylamide/bis-acrylamide (37.5:1), 30% solution and EZ Blue gel staining reagent were obtained from Sigma. Acetic acid (glacial, 99.99+%) was obtained from Aldrich and isopropanol was obtained from Hayman. Biodyne C membrane was obtained from Pall Life Sciences.

Incubations were performed using a PTC-100 Programmable Thermal Controller (MJ Research Inc.).

All solutions were prepared with autoclaved deionised water (WaterPro system, Labconco).

MATERIALS AND METHODS - Electrochemical detection

The following were all obtained from BAS, Congleton, Cheshire UK:

Glassy carbon working electrode (catalogue number MF-2012)
Silver/silver chloride reference electrode (catalogue number MF-2079)
Platinum wire counter (auxiliary) electrode (catalogue number MW-4130)

Low volume cell (catalogue number MF-2040) comprising glass voltammetry vial and glass sample chamber, with replaceable vycor tip.

AutoLab electrochemical workstation (either PGSTAT30 with frequency response analyser or μAutoLab type II) obtained from Windsor Scientific, Slough, Berkshire.

EXAMPLE 1 - Cyclic Voltammetry

This Example describes the cyclic voltammetry method used in Examples 5 to 9 below.

The low volume cell of Figure 1 was filled with approximately 10ml sodium chloride solution (100mM). A 200µl aliquot of the sample for analysis was placed in the glass sample chamber which was then placed in the low volume cell along with the reference and counter electrodes. The electrodes were connected to the Autolab electrochemical workstation and differential pulse voltammetry carried out using the parameters described in Table 1. Prior to analysis the glassy carbon working electrode was polished (using BAS polishing kit catalogue number MF-2060) followed by conditioning. Electrode conditioning consists of cyclic voltammetry, sweeping between ± 1 volt in the appropriate background buffer.

Table 1 Parameters for differential pulse voltammetry:

Parameter:	Anodic sweep
Conditioning potential (V)	0
Conditioning duration (s)	10
Deposition potential (V)	0
Deposition duration (s)	0
Equilibration time (s)	0
Modulation time (s)	0.04
Interval time (s)	0.1
Initial potential (V)	-0.1
End potential (V)	0.7
Step potential (V)	0.003
Modulation amplitude (V)	0.05

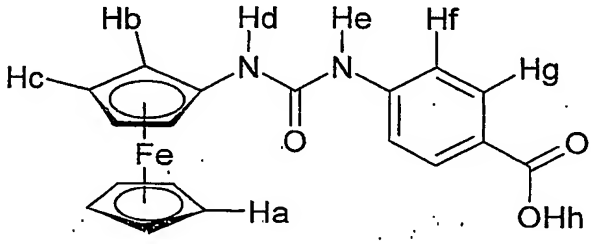
EXAMPLE 2 - Synthesis of *N*-hydroxysuccinimide ester of ferrocenecarboxylic acid

Ferrocenecarboxylic acid (303mg, 1.32mmol) and *N*-hydroxysuccinimide (170mg, 1.47mmol) were dissolved in dioxane (15ml) and added with stirring to a solution of dicyclohexylcarbodiimide (305mg, 1.48mmol) in dioxane (3ml). The mixture was stirred at room temperature for 24 hours during which time a precipitate was formed. The precipitate was removed by filtration, solvent was removed from the filtrate *in vacuo* and the resulting solid purified by silica gel column chromatography, eluting with 8:2 petrol:ethyl acetate. Yield 320mg, 74%.

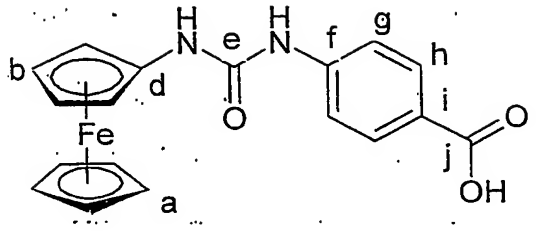
EXAMPLE 3a - Synthesis of ferrocene carbonyl azide

Ferrocene carbonyl azide was prepared from ferrocenecarboxylic acid by reaction with oxalyl chloride and sodium azide.

EXAMPLE 3b - Synthesis of 4-(3'-ferrocenylureido)-1-benzoic acid



Va



Vb

To a purged round-bottom flask was charged ferrocene carbonyl azide (300mg, 1.18 mmol, 1.00 equiv.), 4-aminobenzoic acid (244mg, 1.78 mmol, 1.50 equiv.) and 1,4-dioxane (40 ml) under nitrogen. The reaction mixture was stirred under nitrogen in a 100°C bath for 2 hr 50 min and then allowed to cool to room temperature. 2M HCl (100 ml) was charged to the reaction mixture and the product was extracted into ethyl acetate (150 ml). This phase was washed with 2M HCl (100 ml), dried with sodium sulphate and concentrated *in vacuo* to afford the product. Further drying in a vacuum oven yielded as orange crystals (413mg 96%). ^{1}H-NMR δ (300MHz, d_6-DMSO) 3.96 (2H, b, Hc), 4.14 (5H, s, Ha), 4.53 (2H, b, Hb), 7.54 (2H, m, Hf), 7.85 (2H, m, Hg), 7.98 (1H, s, Hd), 8.87 (1H, s, He) 12.57 (1H, s, Hh), where the position of each hydrogen is as shown in Formula Va. ^{13}C-NMR δ (75.5MHz, d_6-DMSO) 61.0 64.1 66.7 68.1 (Ca,d), 117.2 (Cg), 123.5 (Cj), 130.9 (Ch), 144.6 (Cf), 152.8 (Ce), where the position of each carbon is as shown in Formula Vb.

EXAMPLE 3c - Synthesis of *N*-hydroxysuccinimide ester of 4-(3'-ferrocenylureido)-1-benzoic acid

Dicyclohexylcarbodiimide (DCC) (194 mg, 0.939 mmol, 1.14 equiv.) was dissolved in anhydrous 1,4-dioxane (2 ml) and

charged to a purged round-bottom flask, under nitrogen. *N*-hydroxysuccinimide (108 mg, 0.939 mmol, 1.14 equiv.) was charged. 4-(3'-Ferrocenylureido)-1-benzoic acid (300 mg, 0.823 mmol 1.0 equiv.) was dissolved in anhydrous 1,4-dioxane (13 ml) and charged dropwise to the flask. The solution was stirred at room temperature for 23 hr. A small amount of light brown solid was removed from the red/orange reaction mixture by Buchner filtration. Water (100 ml) and ethyl acetate (50 ml) were charged to the reaction mixture. The ethyl acetate phase was separated and the aqueous was extracted with ethyl acetate (100 ml). The ethyl acetate phases were combined, dried with sodium sulphate and concentrated *in vacuo* to afford the crude product as an orange oil, which was purified using silica flash chromatography with a gradient system from ethyl acetate 60/ petroleum ether (bp 40-60 °C) 40 to ethyl acetate. Drying in a vacuum oven yielded *N*-hydroxysuccinimide ester of 4-(3'-ferrocenylureido)-1-benzoic acid as fine orange crystals (237 mg, 66%). R_f (5:1 ethyl acetate / petroleum ether (bp 40-60 °C) = 0.41 ^{1}H-NMR δ (300MHz, d6-DMSO) 2.88 (4H, s, Hh), 3.98 (2H, t, J = 1.8 Hz, Hc), 4.16 (5H, s, Ha), 4.55 (2H, t, J = 1.8 Hz, Hb), 7.68 (2H, m, Hf), 8.00 (2H, m, Hg), 8.11 (1H, s, Hd), 9.16 (1H, s, He). ^{13}C-NMR δ (75.5MHz, d_6-DMSO) 25.9 (Cl), 61.1 64.2 (Cb and Cc), 69.1 (Ca), 117.7 (Cg), 131.9 (Ch), 170.9 (Ck). MS (FAB+ m/z) 462.07 [M+H].

EXAMPLE 4 - Synthesis of ferrocenylated proteins

The following nomenclature is adopted herein:

- Fc = ferrocene methanoyl group, such that Fc-OH is ferrocene carboxylic acid and Fc-NHR is a ferrocene methyl amido compound.

- FcU = 4-(3′-ferrocenylureido)-1-benzoyl group, such that FcU-OH 4-(3′-ferrocenylureido)-1-benzoic acid and FcU-NHR is a 4-(3′-ferrocenylureido)-1-benzamide compound.
- BSA = Bovine serum albumin

The same general procedure was used for the synthesis of all of the ferrocene labelled proteins. The synthesis of Fc- BSA is described by way of example.

Lyophilised BSA was resuspended in the correct volume of $K_2CO_3/KHCO_3$ buffer (200mM, pH 8.5) to give a BSA concentration of $10mgml^{-1}$. BSA solution (100µl, $10mgml^{-1}$) was added slowly with vortexing to a solution of the *N*-hydroxysuccinimide ester of ferrocenecarboxylic acid in DMSO (100µl, 375mM). The solution was shaken at room temperature for 2 hours, it was then diluted with tris HCl (800µl, 100mM, pH 7.8) and purified using two NAP 10 columns (following the protocol supplied), eluting with firstly with tris HCl (800µl, 100mM, pH 7.8), secondly with deionised water.

BSA concentration was determined by blotting onto Biodyne C membrane using BSA standard concentrations and staining with Ponceau S. Using the method, BSA concentrations were found to be 0.3-0.6 $mgml^{-1}$. Presence of the ferrocene label was confirmed by voltammetric analysis.

FcU-labelled proteins were prepared in an analoguous fashion.

The average number of ferrocene groups present on each labelled BSA molecule was analysed by UV-visible spectroscopy. The UV-visible spectrum of the Fc-BSA conjugate obtained by the procedure described above was

compared with the spectra of Fc/BSA mixtures at various ratios. The overlaid spectra are shown in Figure 2 in which line A is the spectrum for the sample; line B is for BSA:Fc-OH Acid 20:1; line C is for BSA alone; line D is for Fc-OH alone and line E is for BSA:Fc-OH 10:1. From those data, the number of ferrocene molecules present per BSA molecule is estimated to be between 10 and 20.

EXAMPLE 5 - protease assays

Protease assays were performed as follows unless stated otherwise. Lyophilised enzymes were re-suspended to give a concentration of $10mgml^{-1}$. Enzymes were resuspended in the following solutions: HCl (1mM, pH 3.0) (trypsin, α-chymotrypsin, pepsin), NaCl (100mM) (proteinase K, elastase, papain, carboxypeptidase, thermolysin). 75µl of Fc-BSA solution ($0.3-0.6mgml^{-1}$) was used per reaction. Each reaction was carried out in a total volume of 200µl and the reactions were performed in the following buffers (final concentrations are given): 100mM trisHCl pH7.8 (trypsin, α-chymotrypsin, thermolysin, proteinase K); 100mM trisHCl pH 8.5 (elastase); 200mM sodium acetate, 200mM cysteine, 20mM EDTA (papain); 10mM HCl pH 2.0 (pepsin); 25mM trisHCl pH7.5, 500mM NaCl (carboxypeptidase). 2µl enzyme ($10mgml^{-1}$) was added to the 200µl reaction mixture. Samples were incubated at 37°C for 1 hour. The reaction products were analysed by differential pulse voltammetry as described in Example 1.

Data Presentation

Baseline corrected data is displayed as overlayed files in addition to the raw data. Baseline corrected data was obtained using GPES Manager, selecting baseline correction

from the edit data menu, selecting moving average, minimum peak width 0.003V.

EXAMPLE 5A - Digestion of Fc-BSA with trypsin

A digestion of Fc-BSA with trypsin was carried out as described above. The differential pulse voltammogram results are shown in Figure 3 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-trypsin control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 435mV and a peak height of 4.58×10^{-7}A; the no trypsin control reaction has a peak position of 432mV and a peak height of 2.55×10^{-8}A.

As is seen in Figure 3, the current observed at 435mV increases by a factor of 18 upon digestion of the protein to which the electrochemical marker is attached.

EXAMPLE 5B - Digestion of Fc-BSA with α-chymotrypsin

A digestion of Fc-BSA with α-chymotrypsin was carried out as described above. The differential pulse voltammogram results are shown in Figure 4 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-α-chymotrypsin control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 438mV and a peak height of 4.48×10^{-7}A; the no α-chymotrypsin control reaction has a peak position of 432mV and a peak height of 2.55×10^{-8}A.

EXAMPLE 5C - Digestion of Fc-BSA with elastase

A digestion of Fc-BSA with elastase was carried out as described above. The differential pulse voltammogram results are shown in Figure 5 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-elastase control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 430mV and a peak height of 2.57×10^{-7}A; the no elastase control reaction has a peak position of 432mV and a peak height of 2.55×10^{-8}A.

EXAMPLE 5D - Digestion of Fc-BSA with pepsin

A digestion of Fc-BSA with pepsin was carried out as described above. The differential pulse voltammogram results are shown in Figure 6 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-pepsin control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 537mV and a peak height of 8.90×10^{-8}A; the no-pepsin control reaction has a peak position of 522mV and a peak height of 4.19×10^{-8}A.

EXAMPLE 5E - Digestion of Fc-BSA with carboxypeptidase

A digestion of Fc-BSA with carboxypeptidase was carried out as described above. The differential pulse voltammogram results are shown in Figure 7 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-carboxypeptidase control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has

a peak position of 435mV and a peak height of 1.31×10^{-7}A; the no elastase control reaction has a peak position of 427mV and a peak height of 6.86×10^{-8}A.

EXAMPLE 5F - Digestion of Fc-BSA with thermolysin at 37°C

A digestion of Fc-BSA with thermolysin was carried out as described above with the digestion incubation at 37°C. The differential pulse voltammogram results are shown in Figure 8 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-thermolysin control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 429mV and a peak height of 1.62×10^{-8}A; no peak was found in the no-thermolysin control reaction.

EXAMPLE 5G - Digestion of Fc-BSA with thermolysin at 70°C

A digestion of Fc-BSA with thermolysin was carried out as described above with the digestion incubation at 70°C. The differential pulse voltammogram results are shown in Figure 9 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-thermolysin control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 455mV and a peak height of 2.0×10^{-8}A; no peak was found in the no-thermolysin control reaction.

EXAMPLE 5H - Digestion of BSA with trypsin

A digestion of unlabelled BSA with trypsin was carried out in the same manner as for the labelled molecules as described above. The differential pulse voltammogram results are shown

in Figure 10 in which (a) is the trace for a no-trypsin control reaction and (b) is the trace for trypsin BSA reaction. No peaks were found in either reaction product solution.

EXAMPLE 5I - Digestion of FcU-BSA with trypsin

A digestion of FcU-BSA with trypsin was carried out as described above. The differential pulse voltammogram results are shown in Figure 11 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-trypsin control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 97mV and a peak height of 5.01×10^{-7}A; there was no peak in the no-trypsin control reaction.

EXAMPLE 5J - Digestion of FcU-BSA with papain

A digestion of FcU-BSA with papain was carried out as described above. The differential pulse voltammogram results are shown in Figure 12 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-papain control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 93mV and a peak height of 2.62×10^{-7}A; no peak was found in the no-papain control reaction.

EXAMPLE 5K - Digestion of FcU-casein with trypsin

A digestion of FcU-casein with trypsin was carried out as described above. The differential pulse voltammogram results are shown in Figure 13 in which (a) is the trace for the digested product; (b) is the trace for the product of a no-

trypsin control and (c) shows the data from (a) and (b) with baseline correction. The positive reaction has a peak position of 148mV and a peak height of 3.79×10^{-7}A and the no-trypsin control reaction shows has a peak position of 147mV and a peak height of 1.55×10^{-7}A.

EXAMPLE 6 - Variation electrochemical marker signal with enzyme concentration

Five digestions of Fc-BSA with trypsin were carried out using the protocol described above. As described in the general protocol, 2µl of enzyme was used for each 200µl reaction; for reaction (i) 10mg/ml enzyme solution was used; reaction (ii): 1mg/ml; reaction (iii) 0.1mg/ml; reaction (iv) 0.01mg/ml and reaction (v) was a no-trypsin control. The differential pulse voltammogram results are shown in Figure 14 with baseline correction. Reaction (i) has a peak position of 430mV and a peak height of 5.42×10^{-7}A, reaction (ii) has a peak position of 428mV and a peak height of 2.29×10^{-7}A, reaction (iii) has a peak position of 429mV and a peak height of 1.04×10^{-7}A, reaction (iv) has a peak position of 429mV and a peak height of 7.53×10^{-8}A, the no-trypsin control reaction shows has a peak position of 448mV and a peak height of 3.17×10^{-9}A.

As is seen in Figure 14, the magnitude of signal in the differential pulse voltammogram is strongly dependent on the concentration of enzyme present in the digestion experiment. A series of serial dilutions such as those described here may be used to provide data for a calibration standard curve. Such a curve is useful for quantifying enzyme levels or enzyme activities in an experimental sample of unknown protease content.

EXAMPLE 7 - Variation electrochemical marker signal with incubation time

Five digestions of Fc-BSA with trypsin were carried out using the protocol described above. In reaction (i) the incubation at 37° was carried out for 60 minutes, in reaction (ii) for 15 minutes, in reaction (iii) for 5 minutes, in reaction (iv) for 2 minutes and reaction (v) was a 0 minutes no-trypsin control. The differential pulse voltammogram results are shown in Figure 15 with baseline correction. Reaction (i) has a peak position of 435mV and a peak height of 2.49×10^{-7}A, reaction (ii) has a peak position of 429mV and a peak height of 1.88×10^{-7}A, reaction (iii) has a peak position of 435mV and a peak height of 1.57×10^{-7}A, reaction (iv) has a peak position of 428mV and a peak height of 1.04×10^{-7}A, the no-trypsin control reaction shows has a peak position of 460mV and a peak height of 2.11×10^{-8}A.

As is seen in Figure 15, the magnitude of signal in the differential pulse voltammogram is larger the longer the incubation is carried out.

EXAMPLE 8 - The effect of a protease inhibitor on electrochemical marker signal

Five digestions of Fc-BSA with trypsin were carried out using the protocol described above. In addition, soybean trypsin inhibitor was added to reaction mixtures (i) to (iv). Solutions of the soybean trypsin inhibitor were prepared by resuspending inhibitor as supplied in deionised water to a concentration of 10mg/ml and 1mg/ml as appropriate. No inhibitor was added to reaction (i); 0.5µl of 1mg/ml

inhibitor solution was added to reaction (ii); 0.5µl of 10mg/ml inhibitor was added to reaction (iii); 5µl of 10mg/ml inhibitor solution was added to reaction (iv) and 5µl of 10mg/ml inhibitor solution was added to a no-trypsin control reaction. The differential pulse voltammogram results are shown in Figure 16 with baseline correction. Reaction (i) has a peak position of 439mV and a peak height of $2.56x10^{-7}$A, reaction (ii) has a peak position of 435mV and a peak height of $2.12x10^{-7}$A, reaction (iii) has a peak position of 430mV and a peak height of $1.60x10^{-7}$A, reaction (iv) has a peak position of 426mV and a peak height of $5.33x10^{-8}$A, the no-trypsin control reaction shows has a peak position of 429mV and a peak height of $2.75x10^{-8}$A.

As is seen in Figure 16, the magnitude of signal in the differential pulse voltammogram is strongly dependent on the concentration of inhibitor present in the digestion experiment. A series of serial dilutions such as those described here may be used to provide data for a calibration standard curve and such a curve is useful for quantifying inhibitor levels or inhibitor potencies in an experimental sample of unknown protease content. A calibration curve may also find utility in an assay for screening for potential protease inhibitors.

EXAMPLE 9 - Amperometric analysis of digestion reactions

Four digestion reactions were carried out with real-time amperometric analysis. Lyophilised enzymes were re-suspended to give a concentration of $10mgml^{-1}$. Tryspin was resuspended in HCl (1mM, pH 3.0), papain and carboxypeptidase were resuspended in NaCl (100mM). 75µl of Fc-BSA solution (0.3-$0.6mgml^{-1}$) was used per reaction. Each reaction was carried

out in a total volume of 200µl in the following buffers (final concentrations are given): 100mM trisHCl pH7.8 for the trypsin reaction, 20mM EDTA for the papain reaction, and 25mM trisHCl pH7.5, 500mM NaCl for the carboxypeptidase reaction. 2µl enzyme (10mgml^{-1}) was added to the 200µl reaction mixture. The reaction products were analysed by amperometry in an apparatus as described above and shown in Figure 1. The amperometry conditions were as set out in Tables 2 and 3.

Table 2 Parameters for amperometry

Parameter	
Pretreatment	
First conditioning potential (V)	0
Duration (s)	0
Equilibration time (s)	4
Measurement	
Interval time (>0.1s)	0.4
Potential (V)	*
Duration (s)	1200

* The potential applied to the working electrode was dependent on the ferrocenyl label and the pH of the buffer. Working electrode potentials for the enzyme/ferrocenylated substrate combinations are shown in Table 3:

Table 3 Electrode potentials used for amperometric detection of ferrocenyl labels

Enzyme	Ferrocenyl label	Electrode potential*
Trypsin	Fc	0.44
Trypsin	FcU	0.13
Papain	FcU	0.13

The results of the amperometry experiments are shown in Figures 17 and 18.

In Figure 17, there is shown the amperometric time course trace for the trypsin digestion of Fc-BSA. Line A shows the non-faradaic current response to application of the potential to the working electrode before enzyme addition. As the movement of the species present in the solution reaches a steady state, the current response appears to decay. Line B shows faradiac current response over time after addition of the enzyme, which takes place once the non-faradaic current response reaches an equilibrium. The increase in current shown in Line B appears to relate to enzymic digestion of the ferrocene labelled substrate.

In Figure 18, there is shown the amperometric time course trace for the papain digestion of FcU-BSA. Line A shows the non-faradaic current response to application of the potential to the working electrode before enzyme addition. As the movement of the species present in the solution reaches a steady state, the current response appears to decay. Line B shows faradaic current response over time after addition of the enzyme which takes place once the non-faradaic current response reaches an equilibrium. The increase in current shown in Line B appears to relate to enzymic digestion of the ferrocene labelled substrate.

The amperometric time course trace for the trypsin digestion of FcU-BSA is not illustrated in the drawings but was analogous.

As is seen from figures 17 and 18, the electrochemically active marker allows a protease reaction to be followed in real-time as the reaction progresses without it being necessary to withdraw aliquots. A large amount of kinetic data may be extracted from plots such as those in figures 17 and 18, enabling enzyme kinetics to be studied.

CLAIMS

1. A method of detecting protease activity in a sample solution comprising contacting the sample solution with a protease substrate labelled with an electrochemically active marker, providing conditions under which any protease which may be present in the sample may degrade the protease substrate and electrochemically determining information relating to the electrochemically active marker.

2. A method as claimed in claim 1 wherein the information relating to the electrochemically active marker is obtained by voltammetry.

3. A method as claimed in claim 2 wherein the information relating to the electrochemically active marker is obtained by differential pulse voltammetry.

4. A method as claimed in claim 1 wherein the information relating to the electrochemically active marker is obtained by an amperometric technique.

5. A method as claimed in any one of claims 1 to 4 wherein the information relating to the electrochemically active marker is obtained by a technique that utilises one or more electrodes that are functionally surrounded by a selectively permeable membrane.

6. A method as claimed in any one of claims 1 to 5 wherein the electrochemically active marker is a metallocene moiety.

7. A method as claimed in claim 6 wherein the electrochemically active marker is a ferrocene moiety.

8. A method as claimed in any one of claims 1 to 7 wherein the electrochemically active marker is attached to the substrate through a linker.

9. A method as claimed in any one of claims 1 to 8 wherein each substrate molecule is, on average, labelled with more than one electrochemically active marker molecule.

10. Use of a method as described in any one of claims 1 to 9 for detecting a disease in a subject.

11. Use of a method as described in any one of claims 1 to 9 for detecting a pathogen.

12. Use of a method as described in any one of claims 1 to 9 for screening for a protease inhibitor.

13. A protease or proteinase assay kit comprising a protease substrate labelled with an electrochemically active marker.

14. An apparatus arranged to carry out a method as described in any one or more of claims 1 to 9.

15. A compound of formula III,

$$Mc\text{-}NR'\text{-}C(=O)\text{-}X\text{-}(Ar)_n\text{-}(L)_m\text{-}R \qquad III$$

Wherein

- Mc is a metallocenyl group in which each ring may independently be substituted or unsubstituted,

- the metallocenyl group comprises a metal ion M selected from the group consisting of iron, chromium, cobalt, osmium, ruthenium, nickel or titanium,
- R' is H or lower alkyl,
- X is either NR' or O,
- Ar is a substituted or unsubstituted aryl group,
- n is 0 or 1,
- L is a linker group,
- m is 0 or 1, and
- R is a protein or peptide.

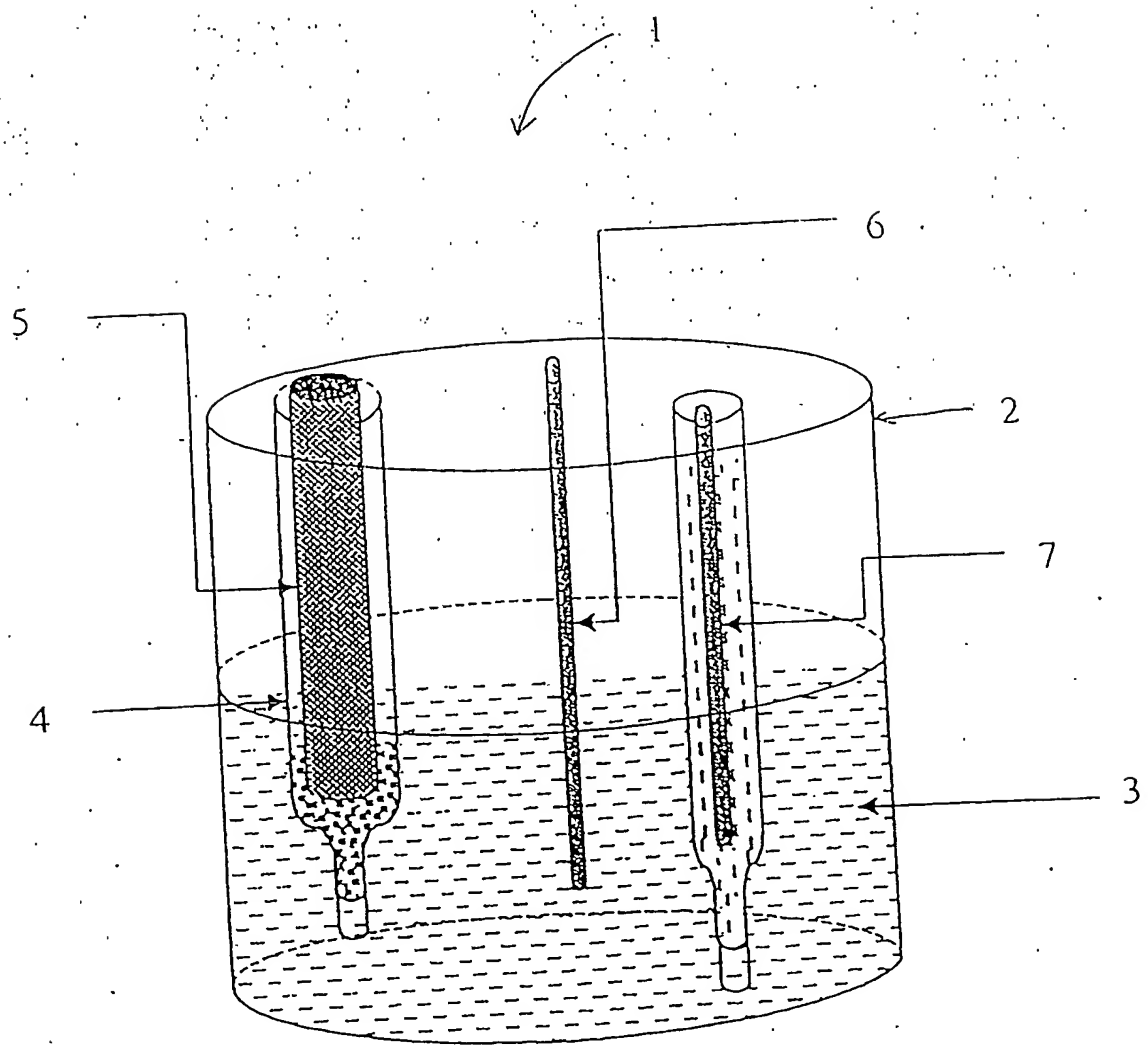


Fig. 1

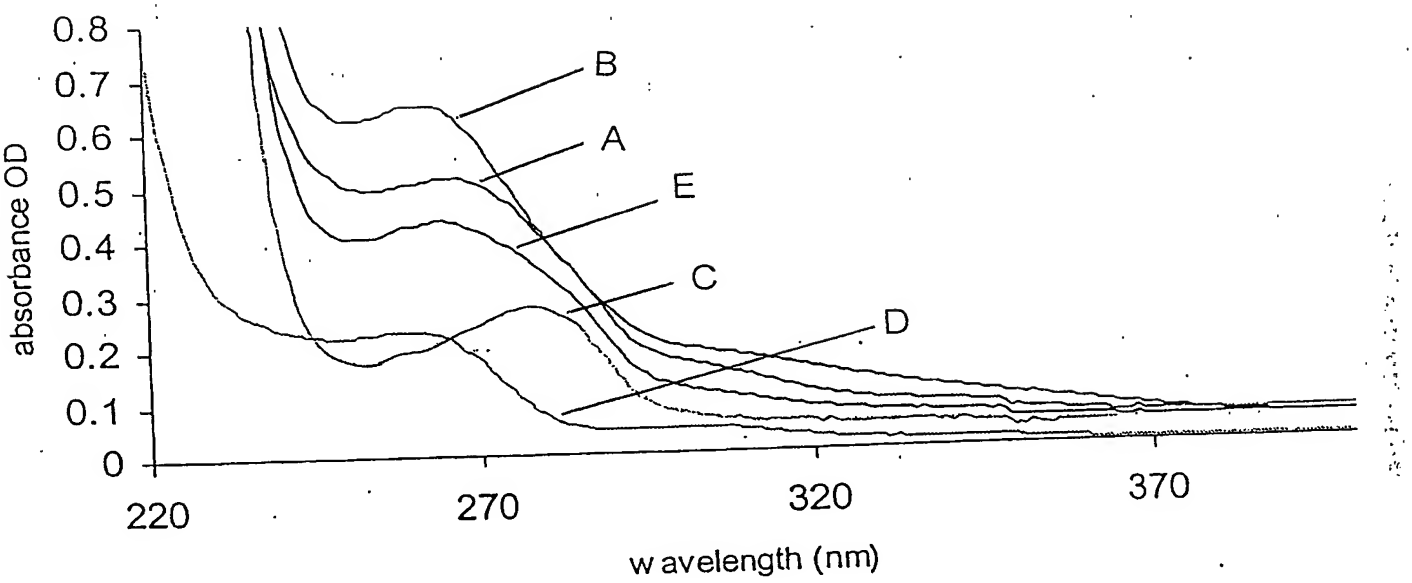


Fig. 2

A = Fc:BSA sample B = BSA:FCA 20:1 C= BSA D = FCA E = BDSA:FCA 10:1

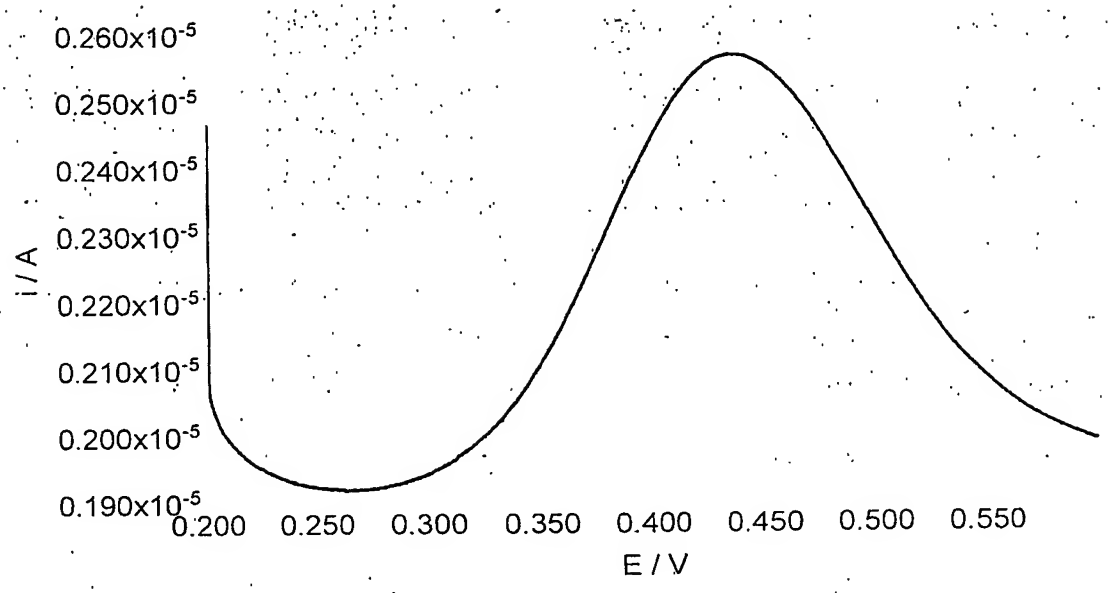


Figure 3(a)

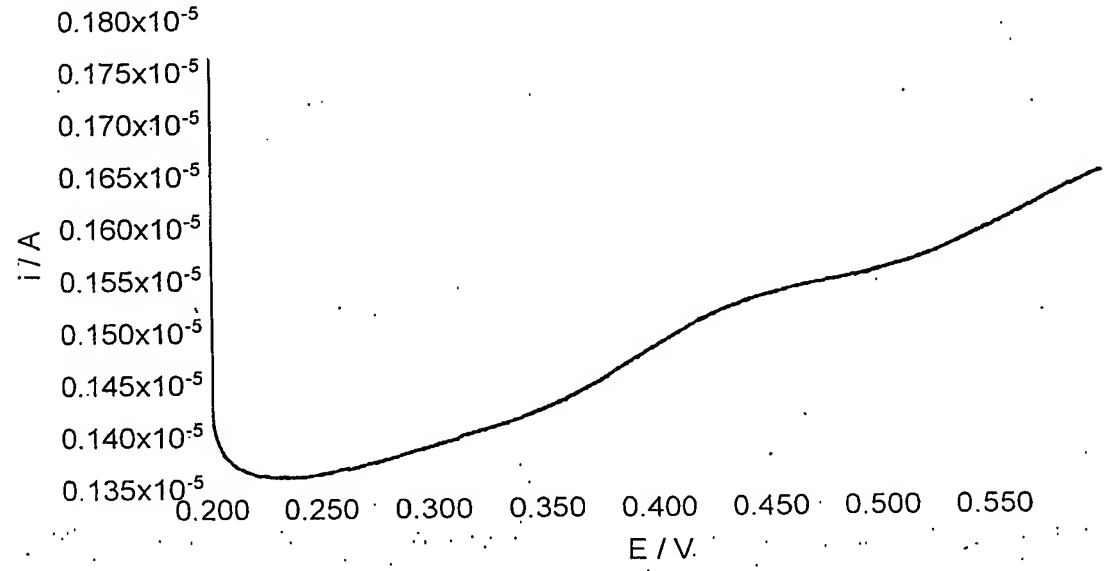
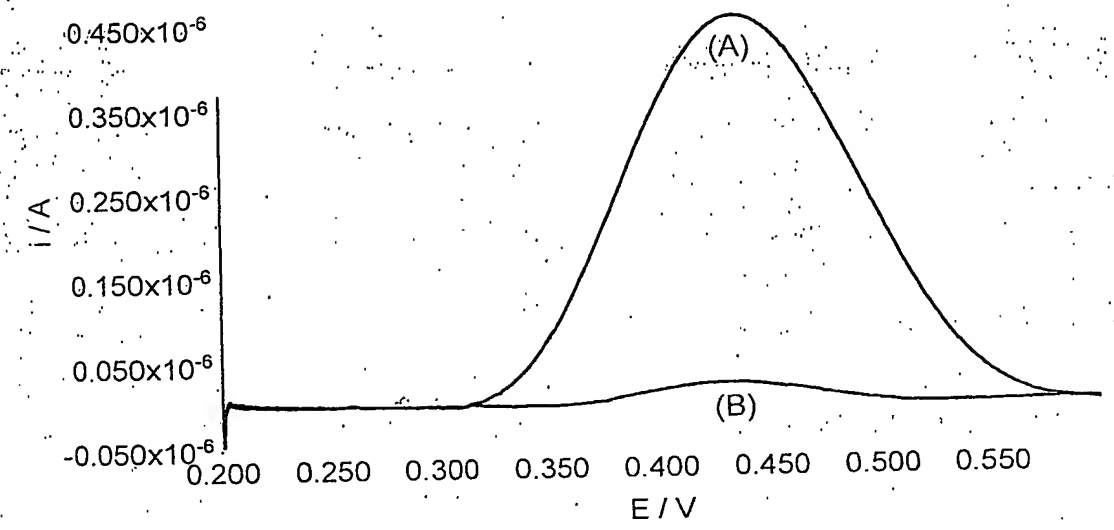


Figure 3(b)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0.350x10-6
0.250x10-6
0.150x10-6
0.050x10-6
-0.050x10-6
i / A
(A)
(B)
0.200
0.250
0.300
0.350
0.400
0.450
0.500
0.550
E / V

Figure 3(c)

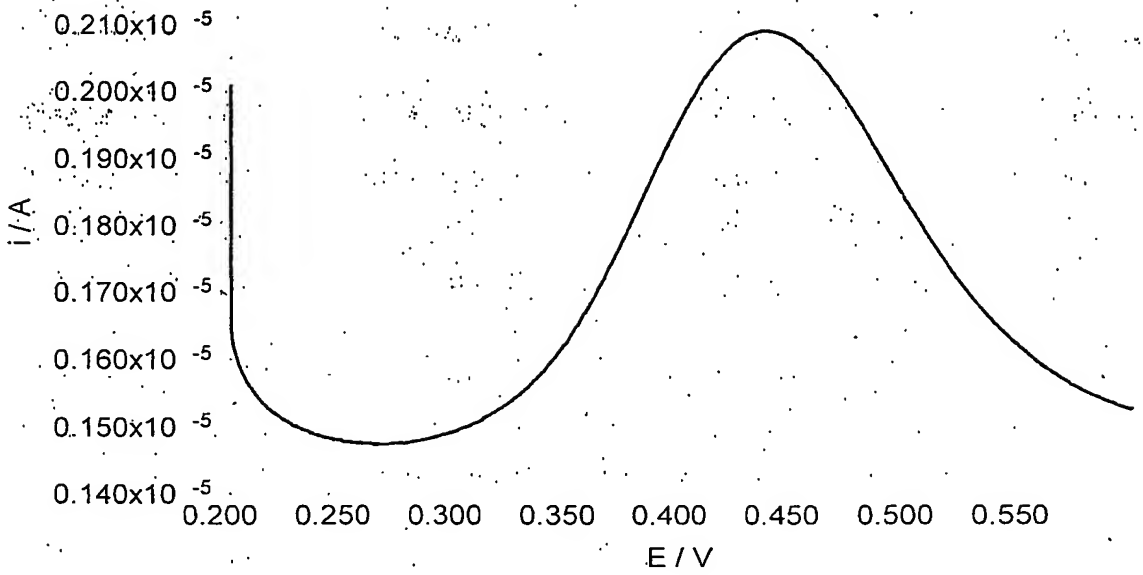


Figure 4(a)

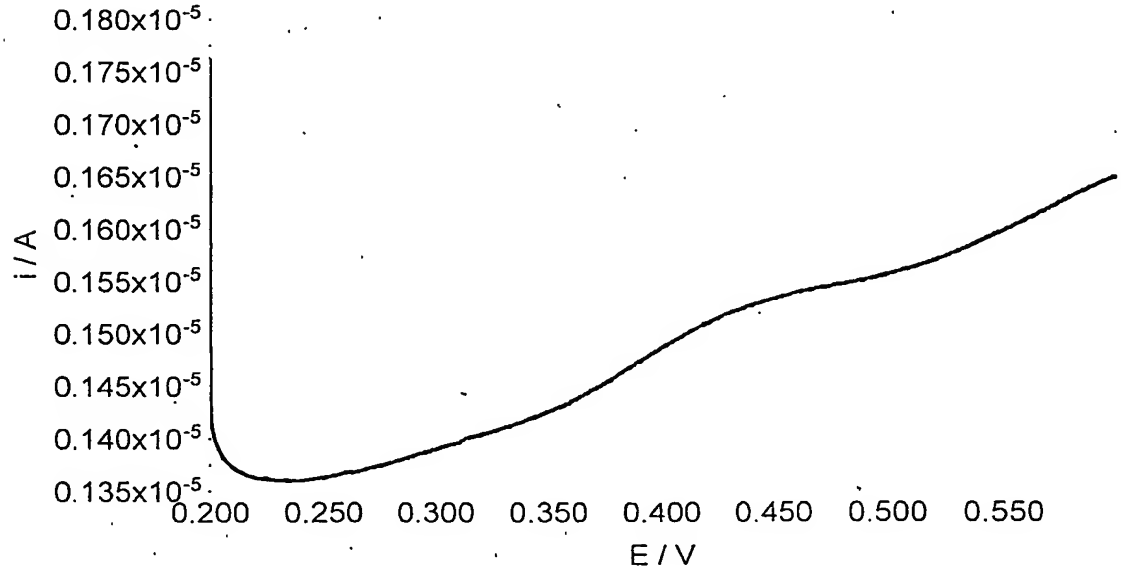
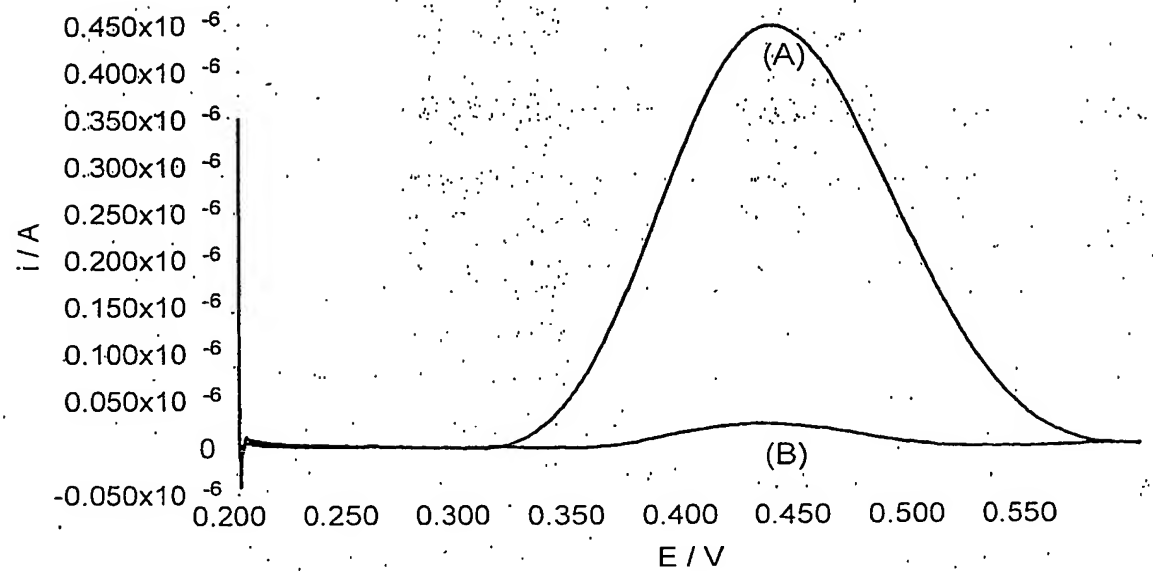


Figure 4(b)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0.350x10 -6
0.300x10 -6
0.250x10 -6
0.200x10 -6
0.150x10 -6
0.100x10 -6
0.050x10 -6
0
-0.050x10 -6
i / A
(A)
(B)
0.200
0.250
0.300
0.350
0.400
0.450
0.500
0.550
E / V

Figure 4(c)

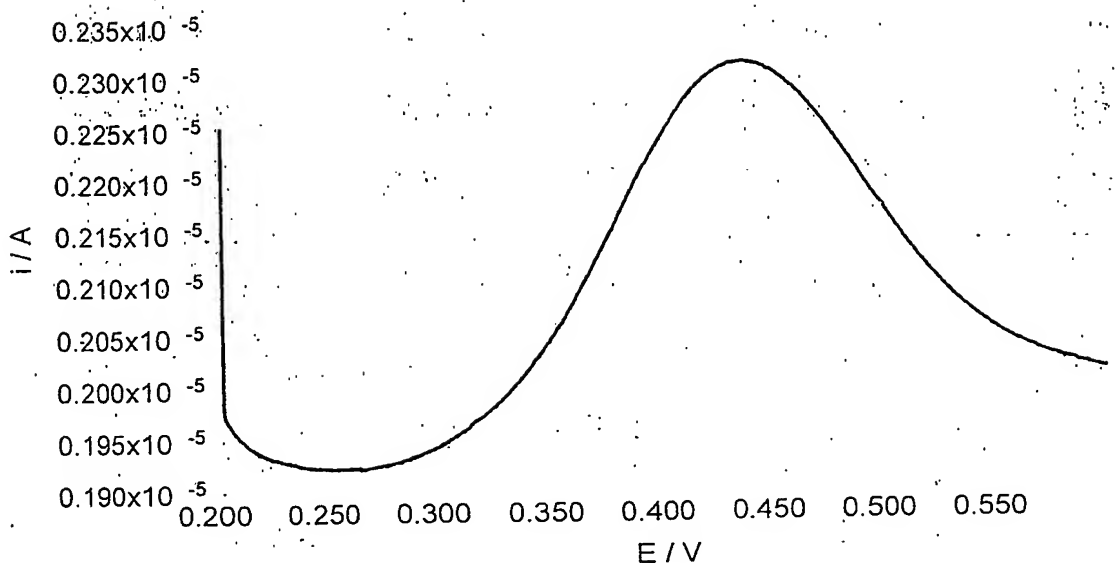


Figure 5(a)

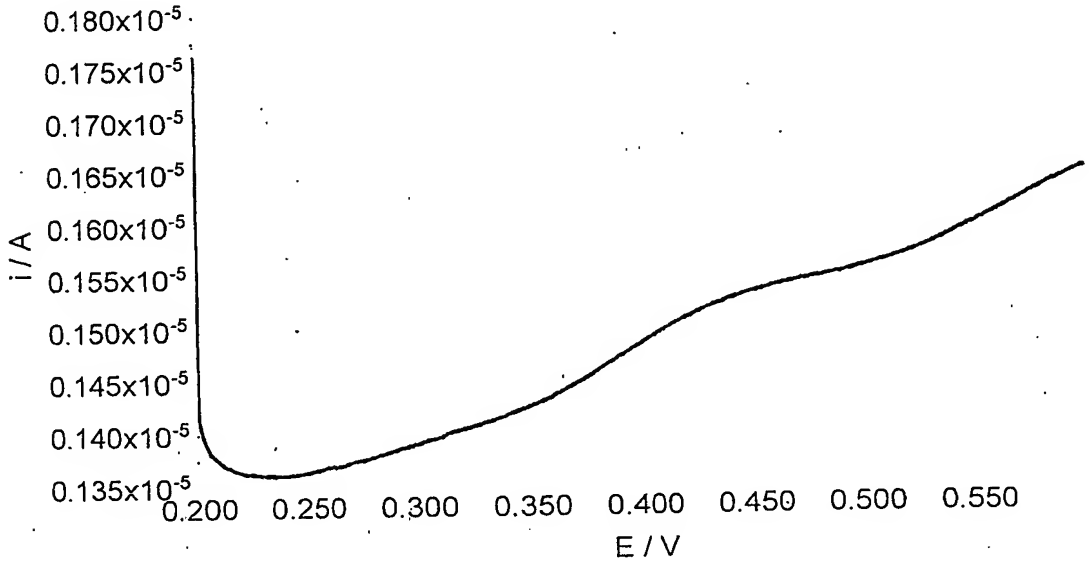
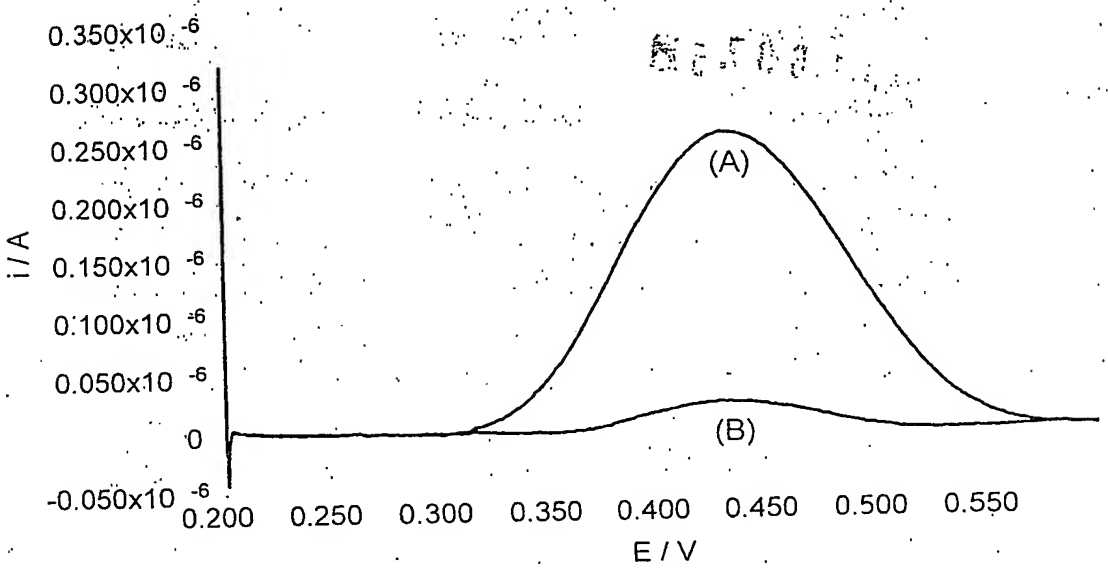


Figure 5(b)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0.250x10 -6
0.200x10 -6
0.150x10 -6
0.100x10 -6
0.050x10 -6
0
-0.050x10 -6
i / A
(A)
(B)
0.200
0.250
0.300
0.350
0.400
0.450
0.500
0.550
E / V

Figure 5(c)

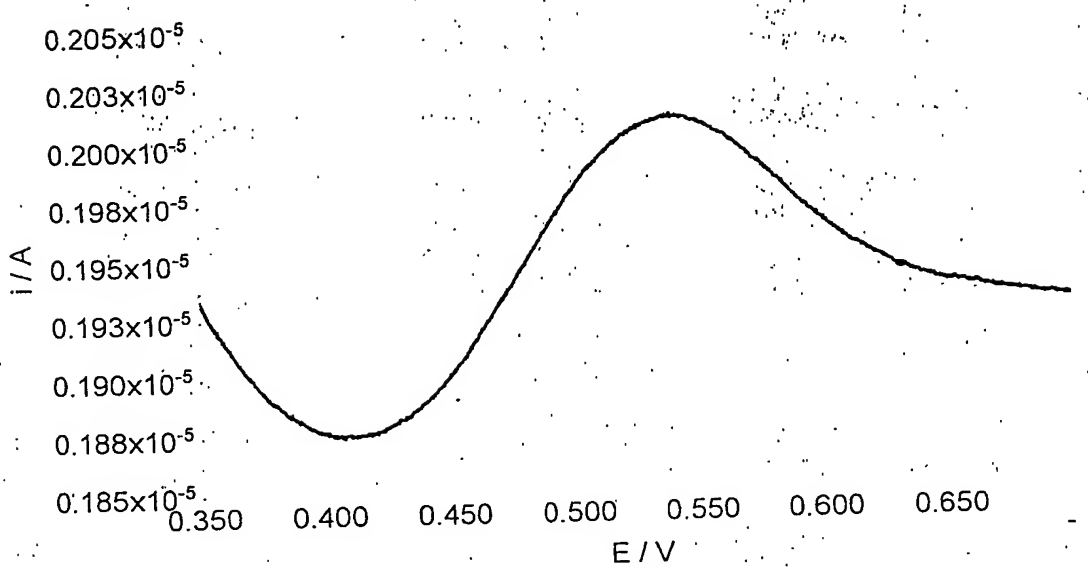


Figure 6(a)

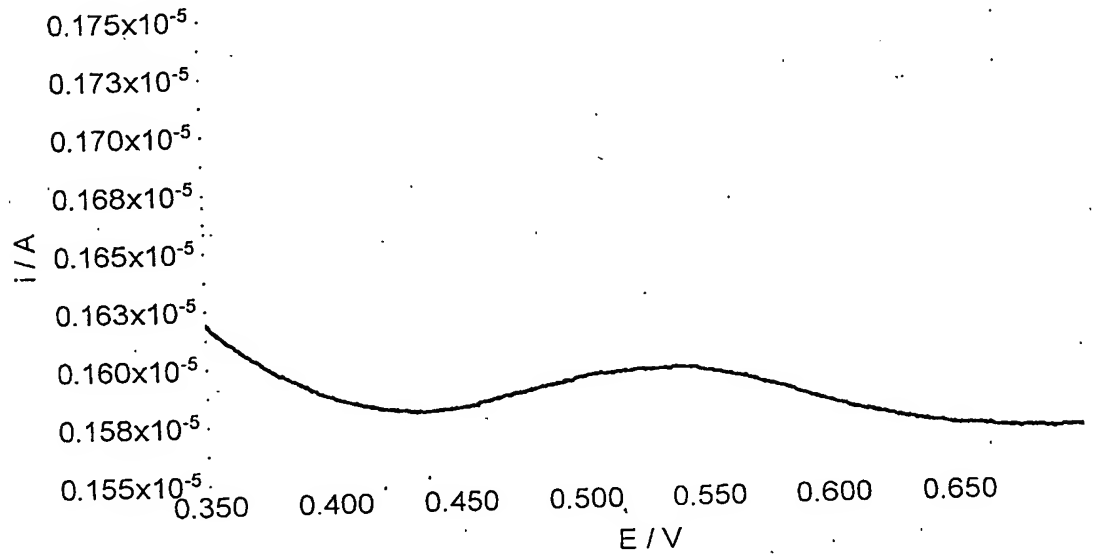


Figure 6(b)

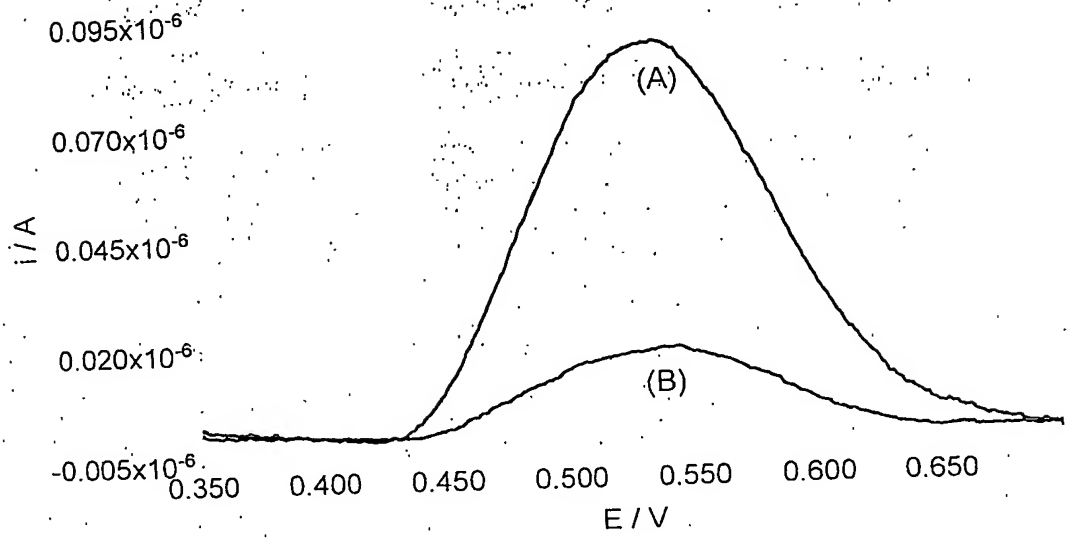

0.095x10-6
0.070x10-6
0.045x10-6
0.020x10-6
-0.005x10-6
i / A
(A)
(B)
0.350
0.400
0.450
0.500
0.550
0.600
0.650
E / V

Figure 6(c)

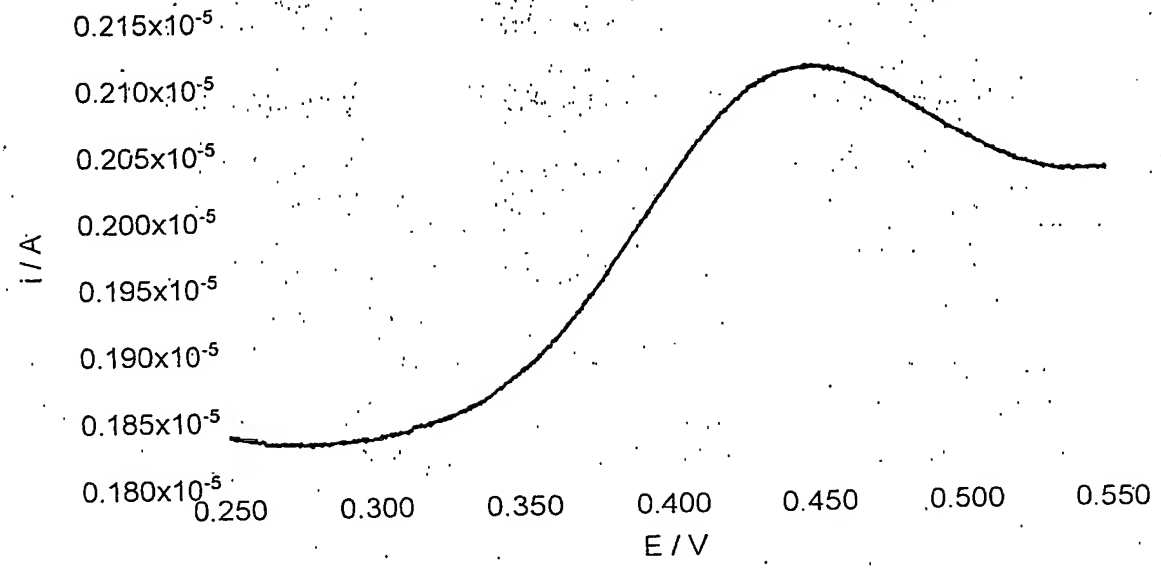


Figure 7(a)

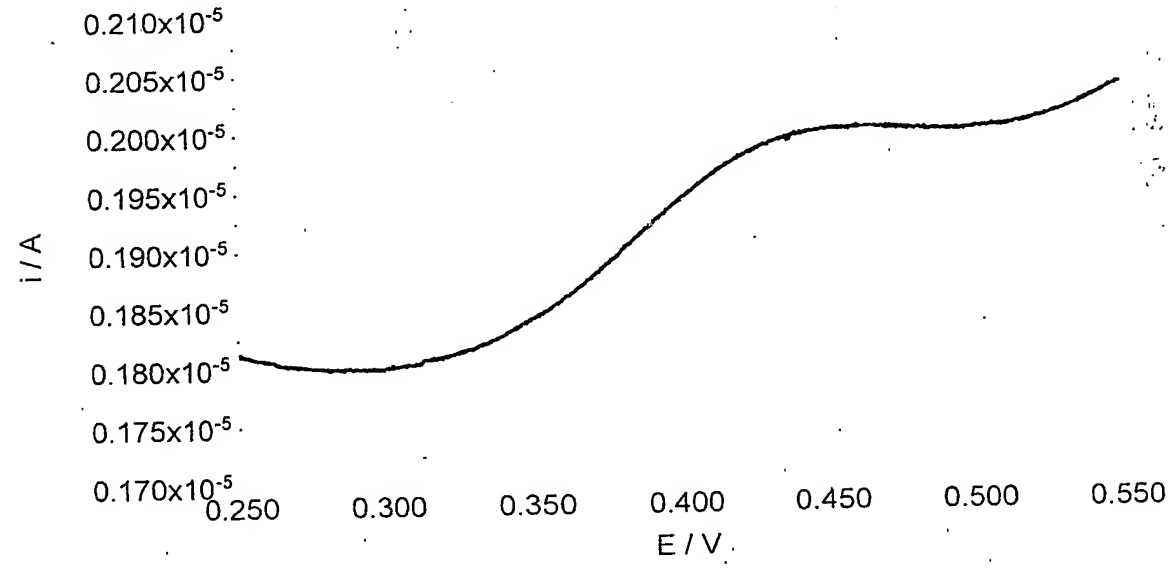
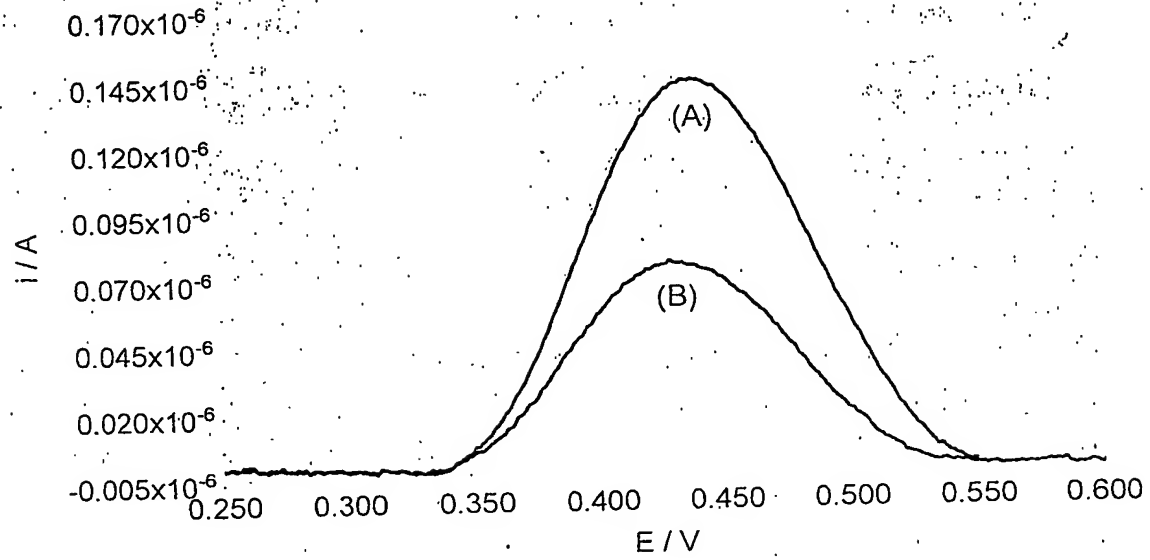


Figure 7(b)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0.120x10-6
0.095x10-6
0.070x10-6
0.045x10-6
0.020x10-6
-0.005x10-6
i / A
(A)
(B)
0.250
0.300
0.350
0.400
0.450
0.500
0.550
0.600
E / V

Figure 7(c)

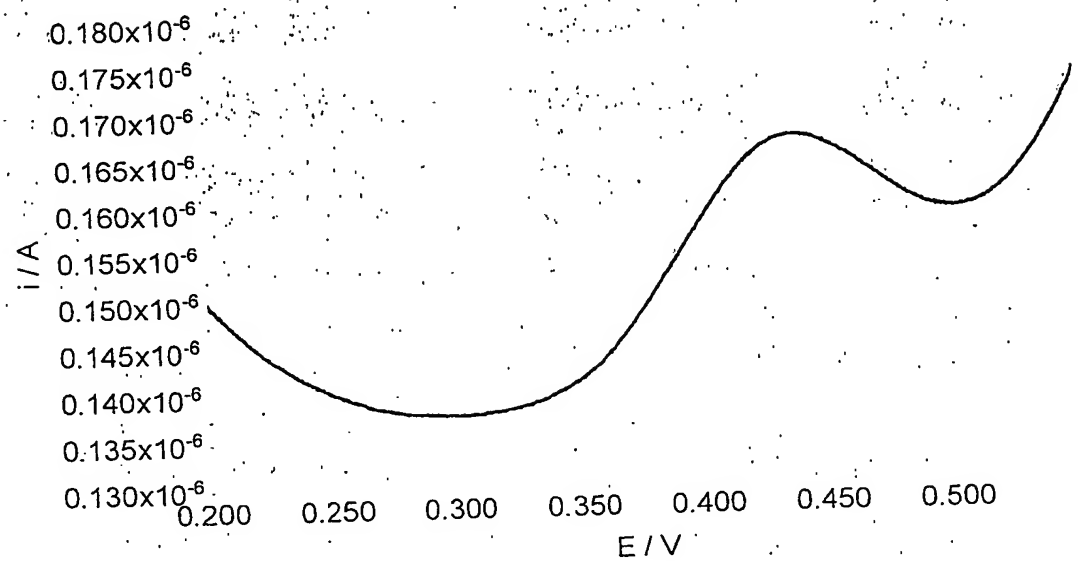


Figure 8(a)

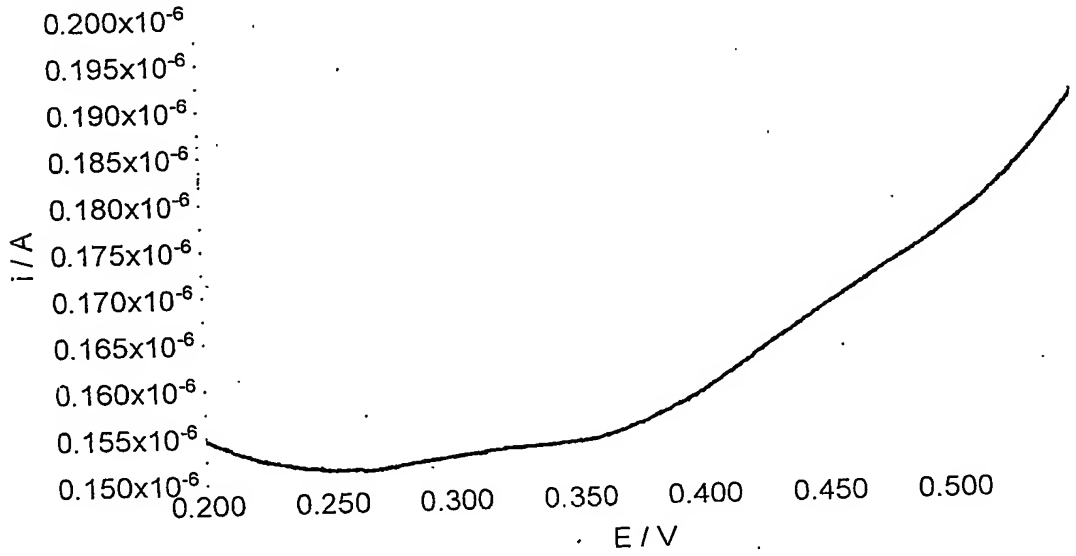
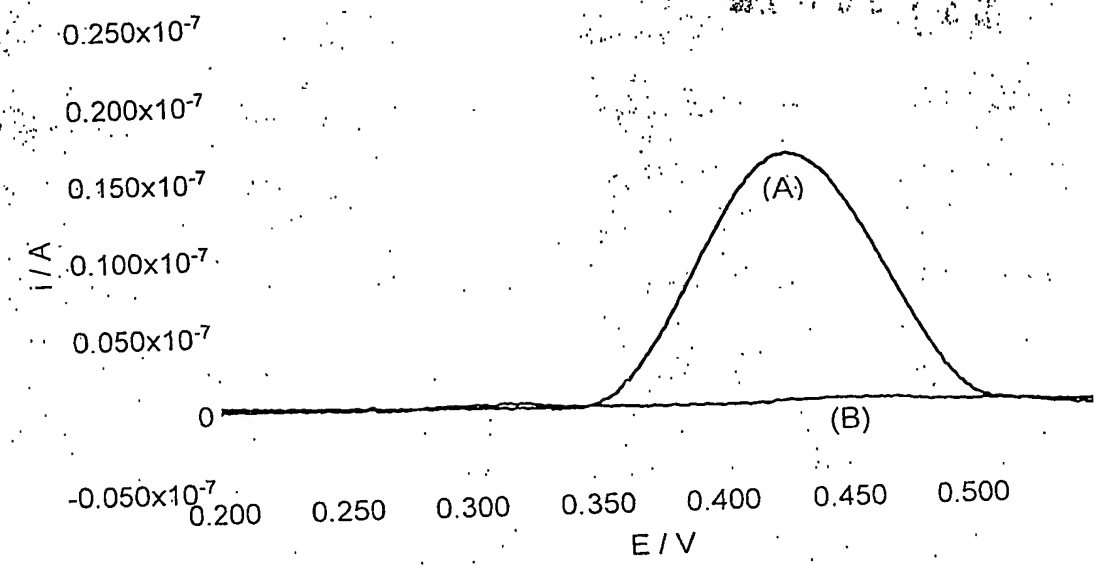


Figure 8(b)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0.150x10-7
0.100x10-7
0.050x10-7
0
-0.050x10-7
i / A
(A)
(B)
0.200
0.250
0.300
0.350
0.400
0.450
0.500
E / V

Figure 8(c)

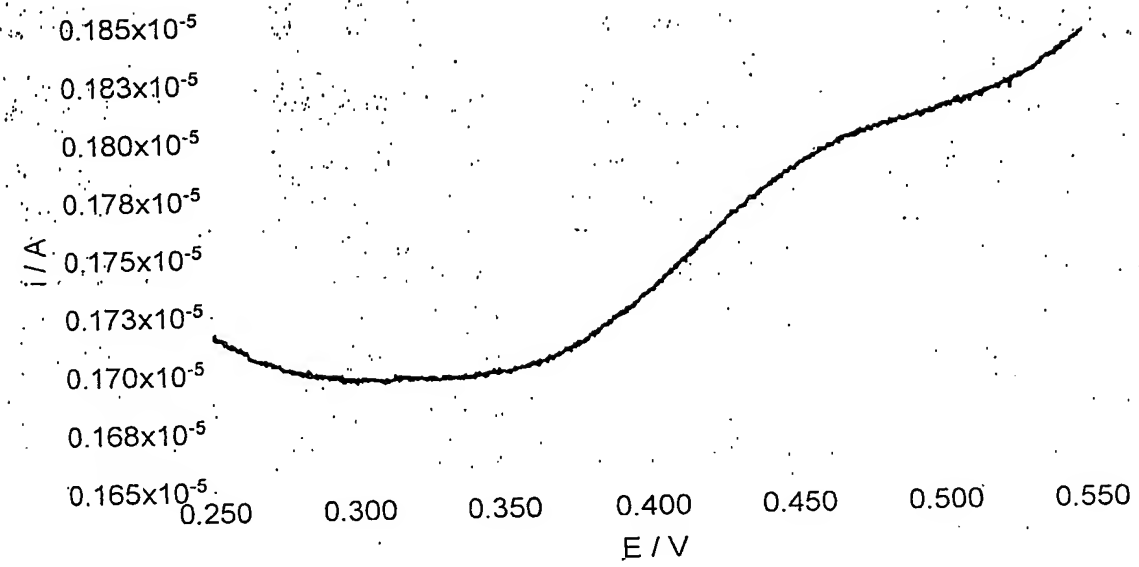


Figure 9(a)

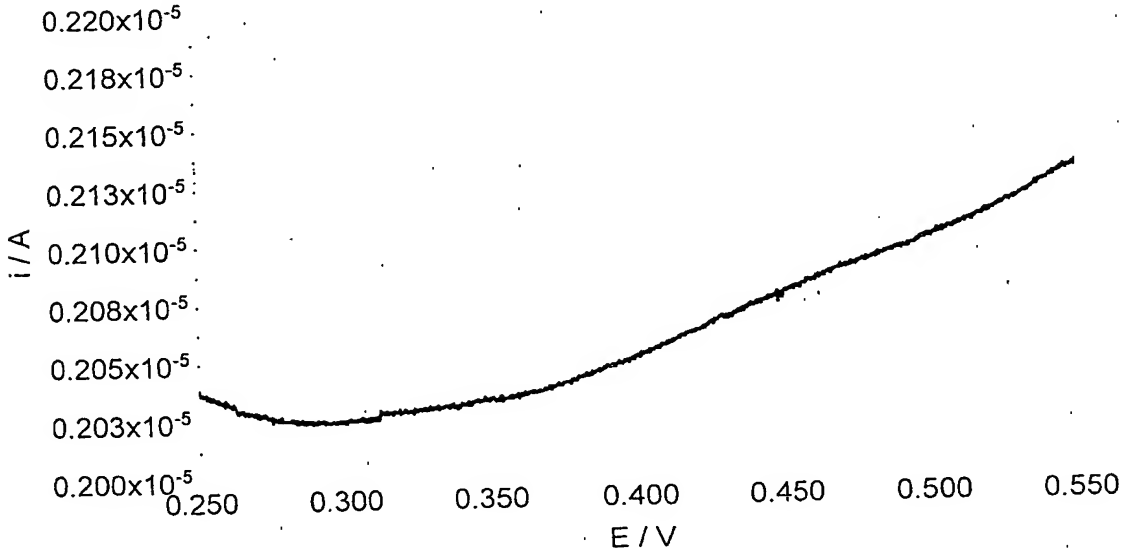
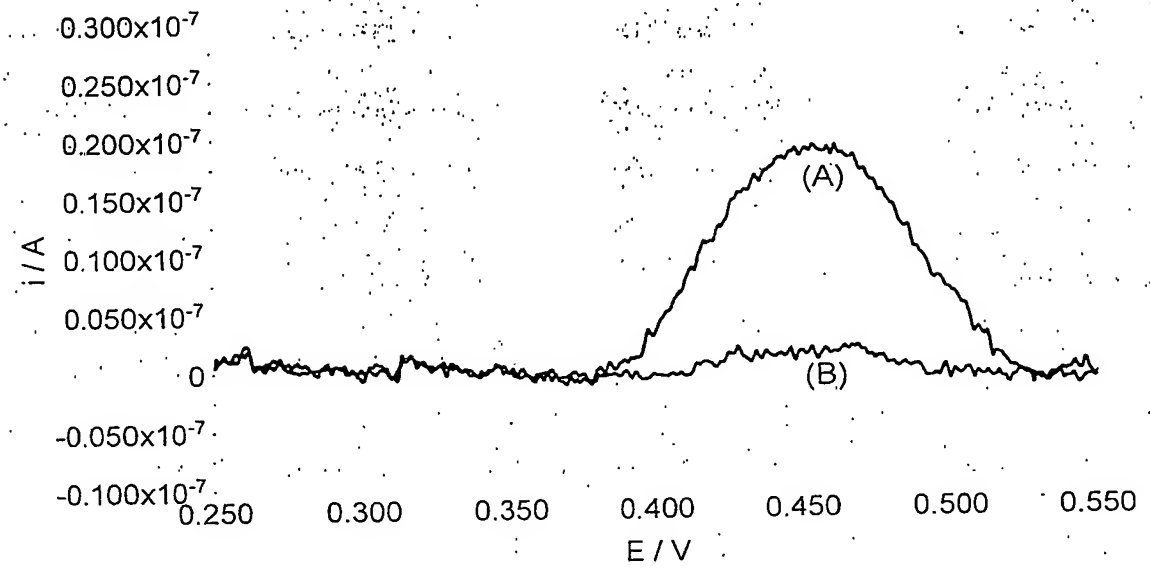


Figure 9(b)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0.150x10-7
0.100x10-7
0.050x10-7
0
-0.050x10-7
-0.100x10-7
i / A
(A)
(B)
0.250
0.300
0.350
0.400
0.450
0.500
0.550
E / V

Figure 9(c)

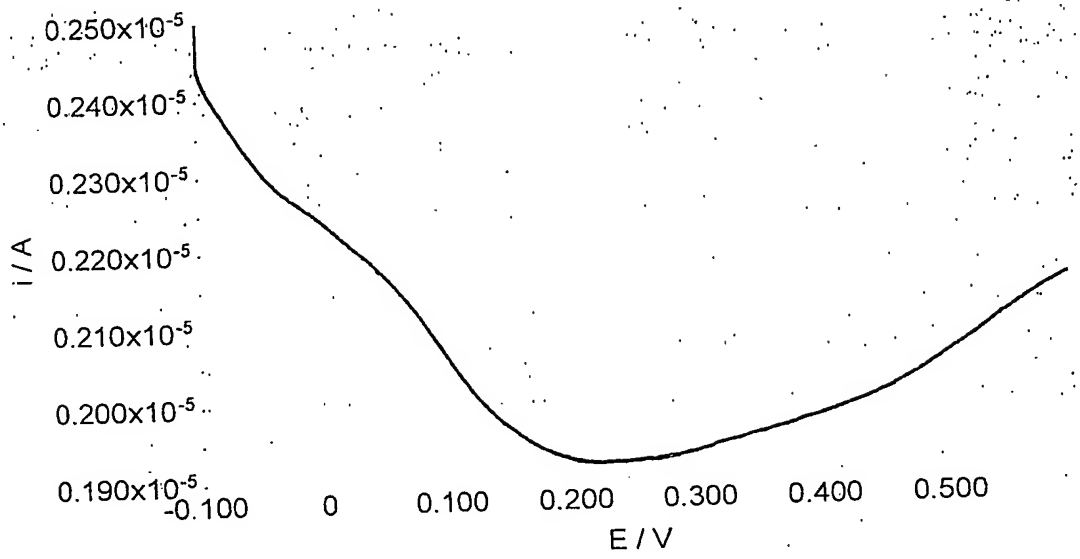


Figure 10(a)

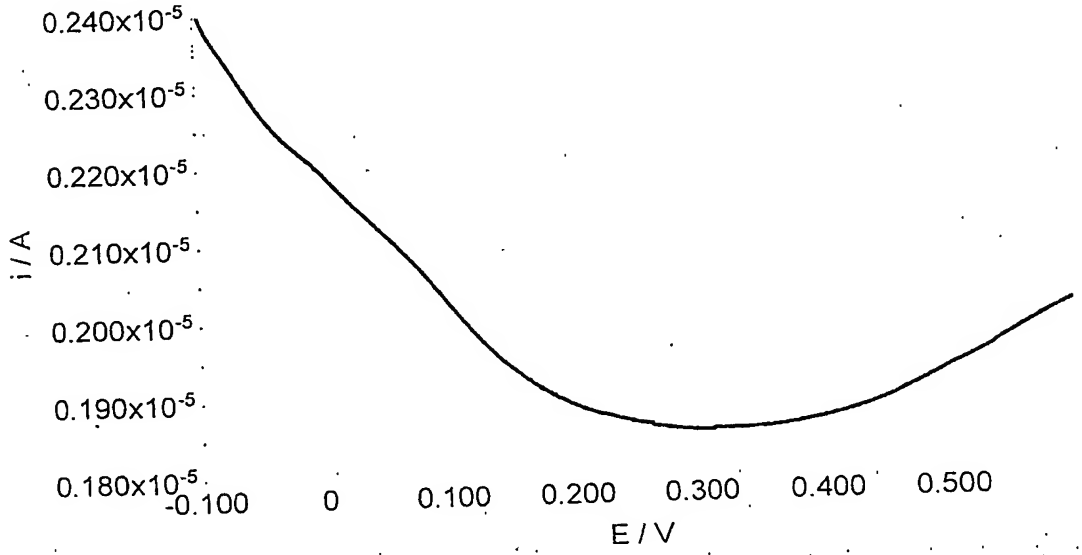


Figure 10(b)

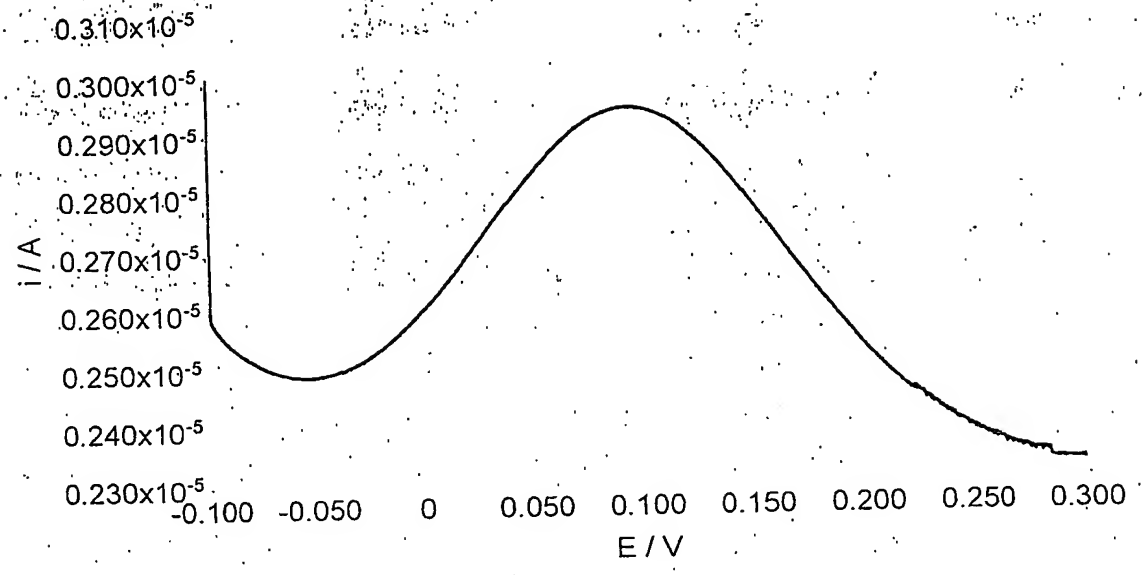


Figure 11(a)

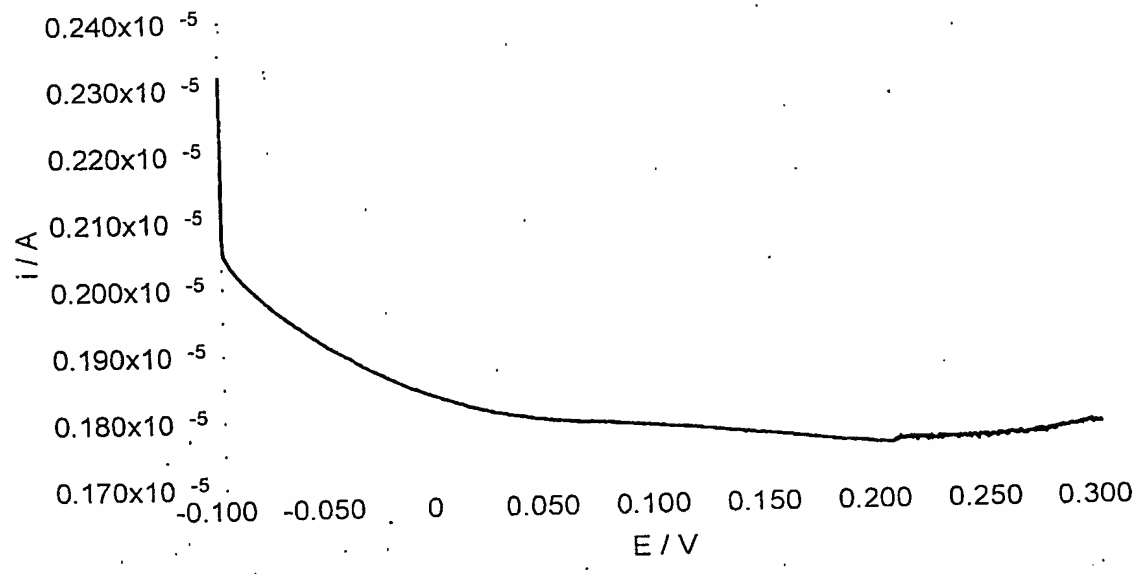
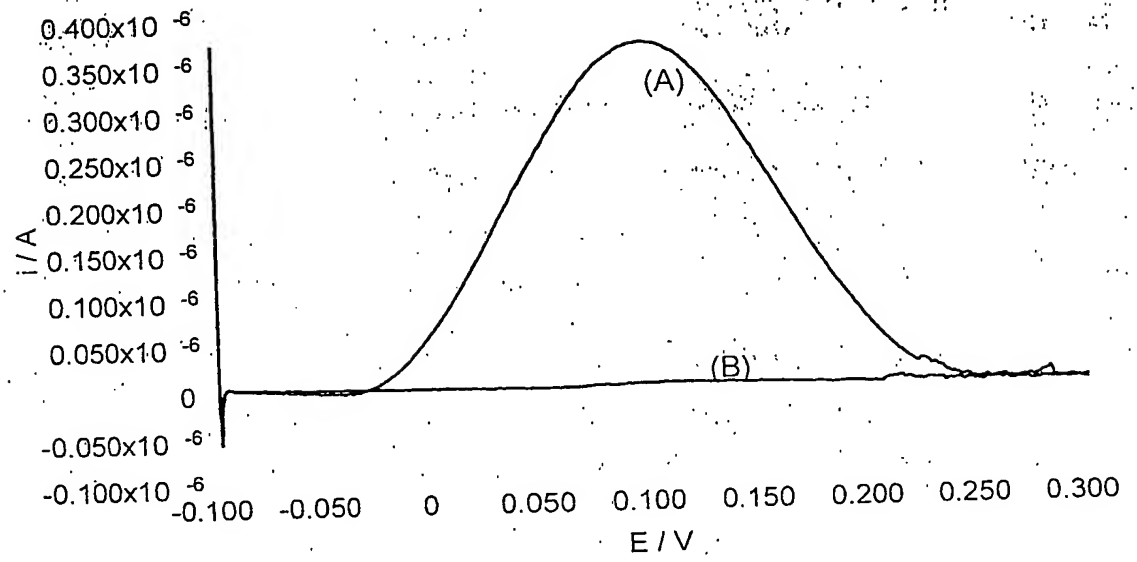


Figure 11(b)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0.150x10 -6
0.100x10 -6
0.050x10 -6
0
-0.050x10 -6
-0.100x10 -6
i / A
(A)
(B)
-0.100
-0.050
0
0.050
0.100
0.150
0.200
0.250
0.300
E / V

Figure 11(c)

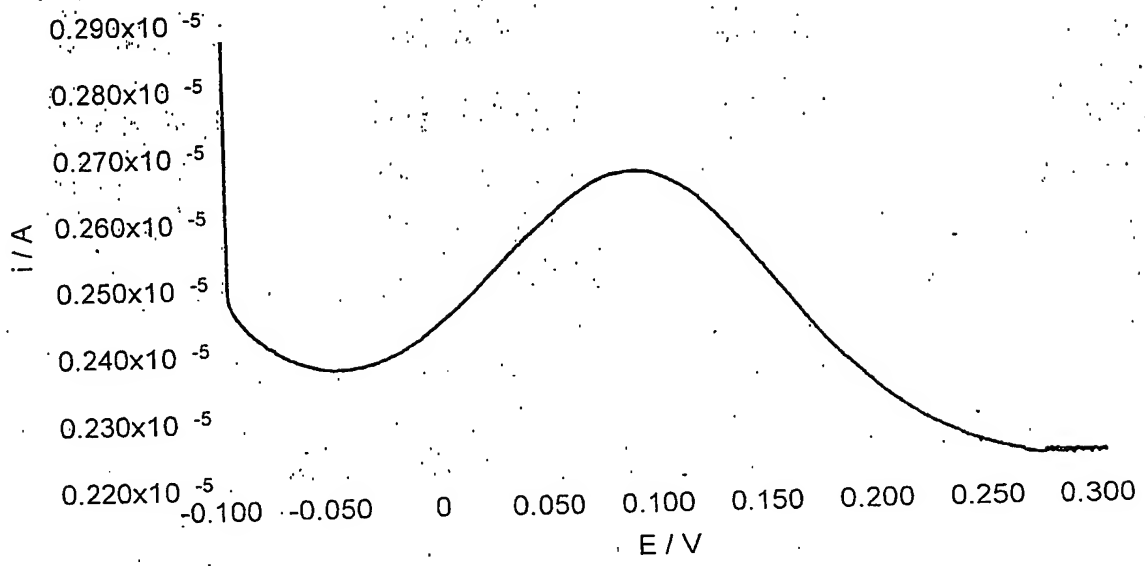


Figure 12(a)

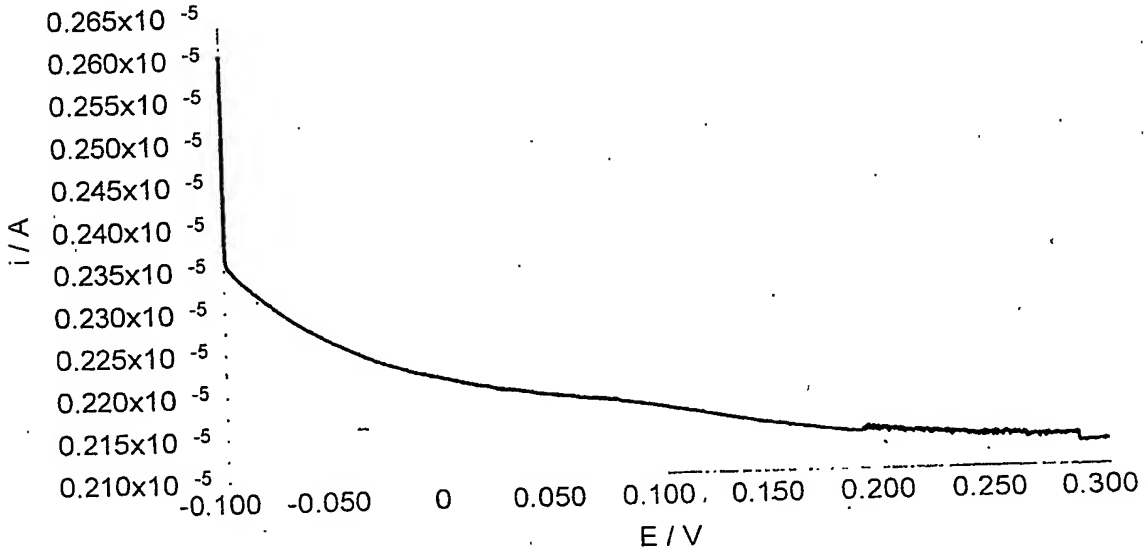
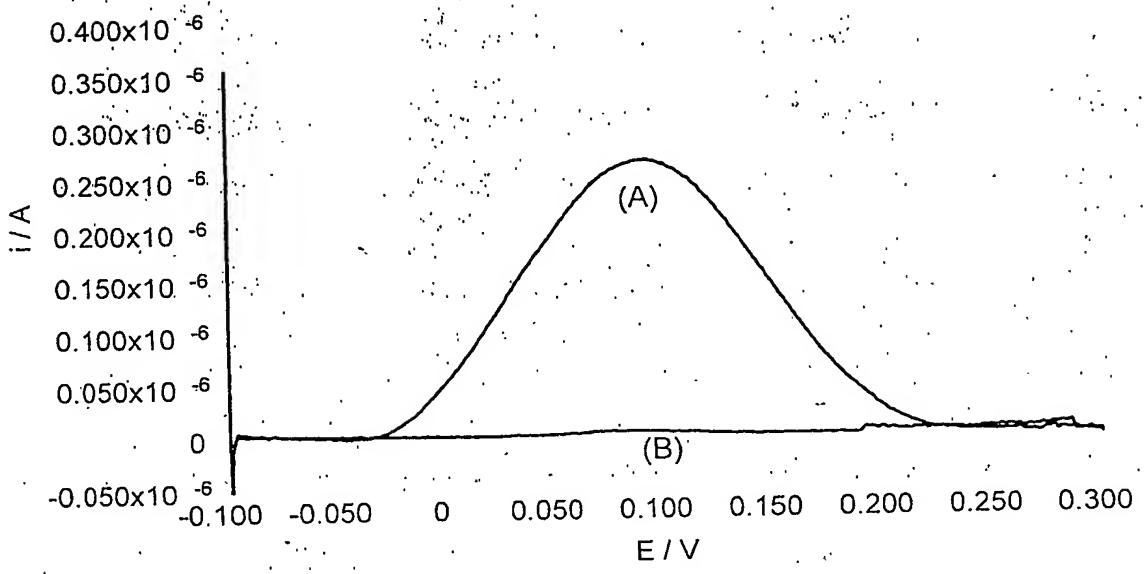


Figure 12(b)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0.150x10 -6
0.100x10 -6
0.050x10 -6
0
-0.050x10 -6
i / A
(A)
(B)
-0.100
-0.050
0
0.050
0.100
0.150
0.200
0.250
0.300
E / V

Figure 12(c)

6. Trypsin digestion of ferrocenylated casein

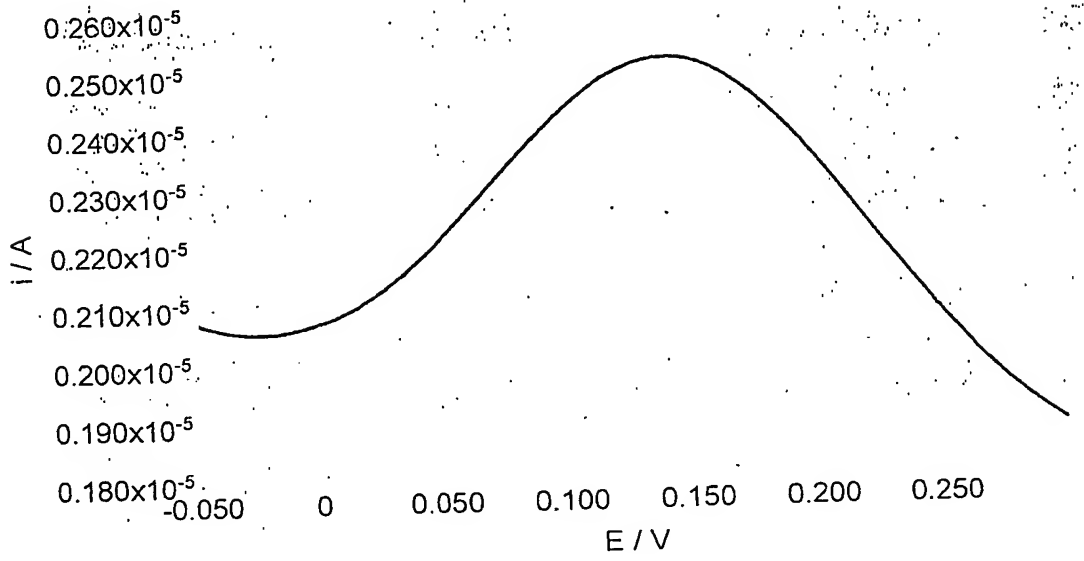


Figure 13(a)

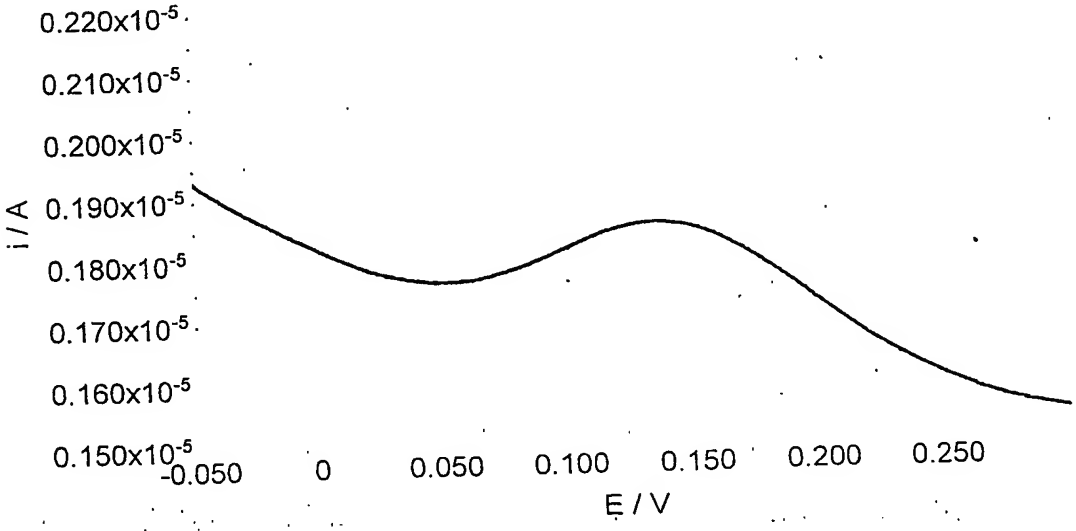
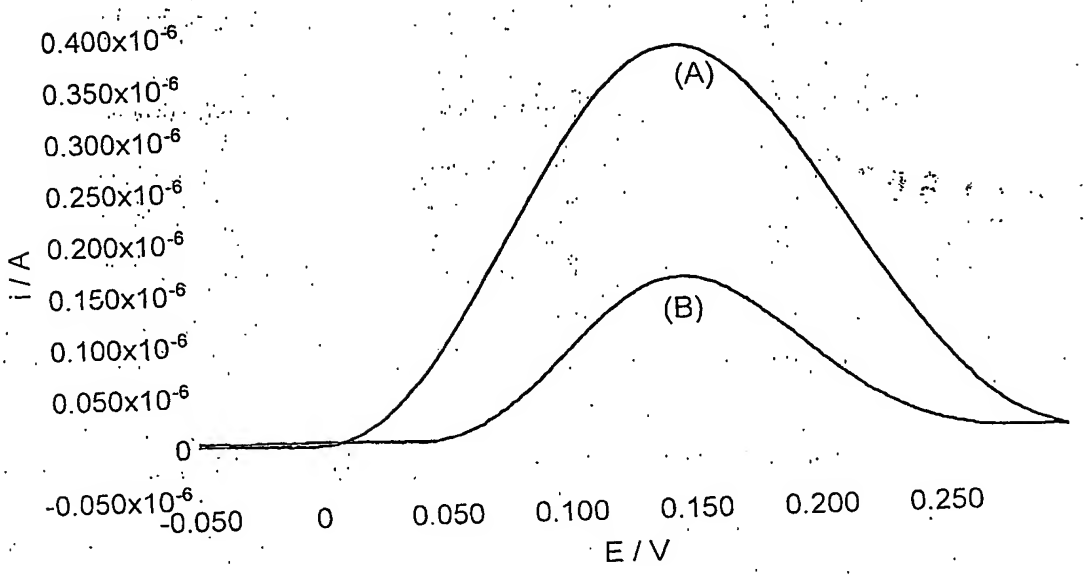


Figure 13(b)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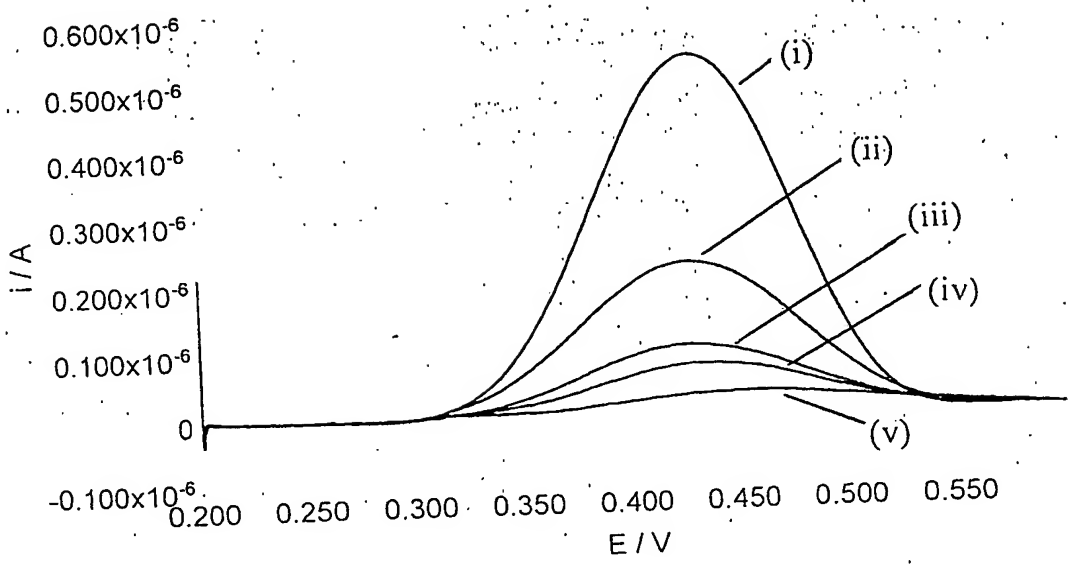
0.300x10-6
0.250x10-6
0.200x10-6
0.150x10-6
0.100x10-6
0.050x10-6
0
-0.050x10-6
i / A
(A)
(B)
-0.050
0
0.050
0.100
0.150
0.200
0.250
E / V

Figure 13(c)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0.500x10-6
0.400x10-6
0.300x10-6
0.200x10-6
0.100x10-6
0
-0.100x10-6
i / A
(i)
(ii)
(iii)
(iv)
(v)
0.200
0.250
0.300
0.350
0.400
0.450
0.500
0.550
E / V

Figure 14

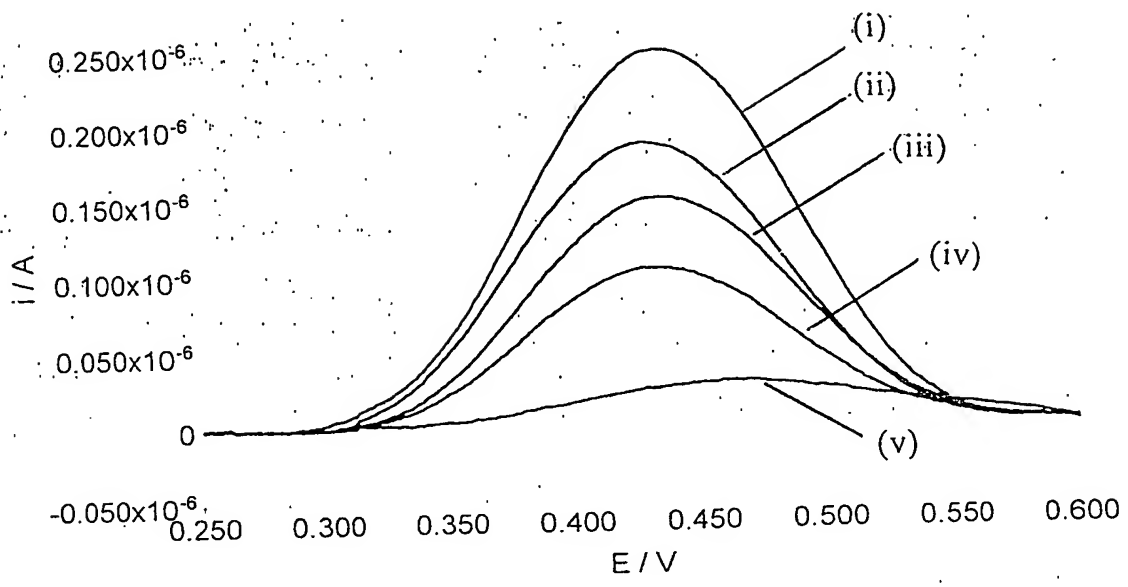
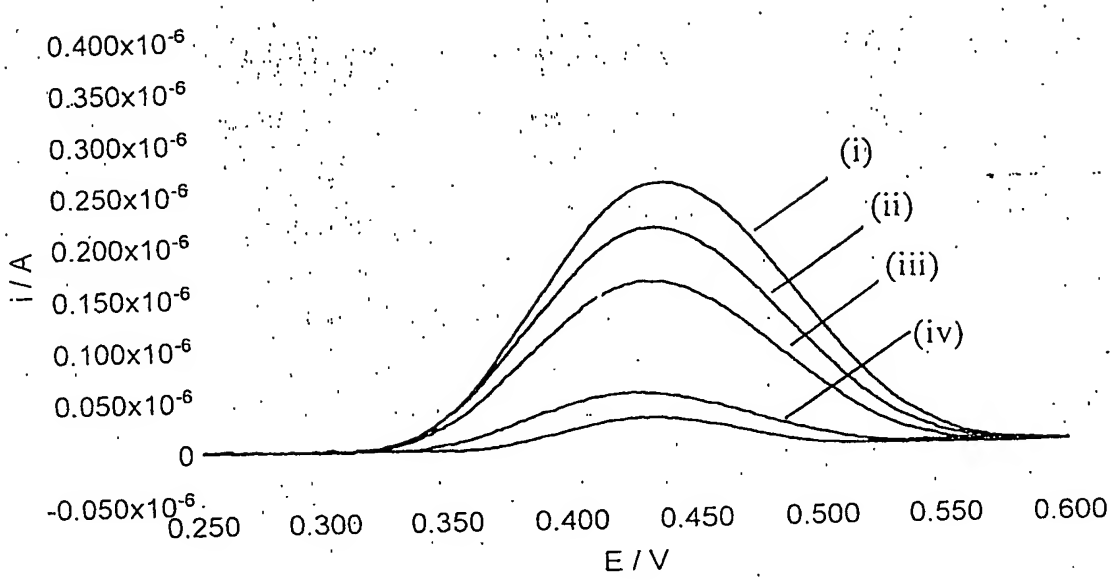

(i)
(ii)
(iii)
(iv)
(v)
0.250x10-6
0.200x10-6
0.150x10-6
0.100x10-6
0.050x10-6
0
-0.050x10-6
i / A
0.250
0.300
0.350
0.400
0.450
0.500
0.550
0.600
E / V

Figure 15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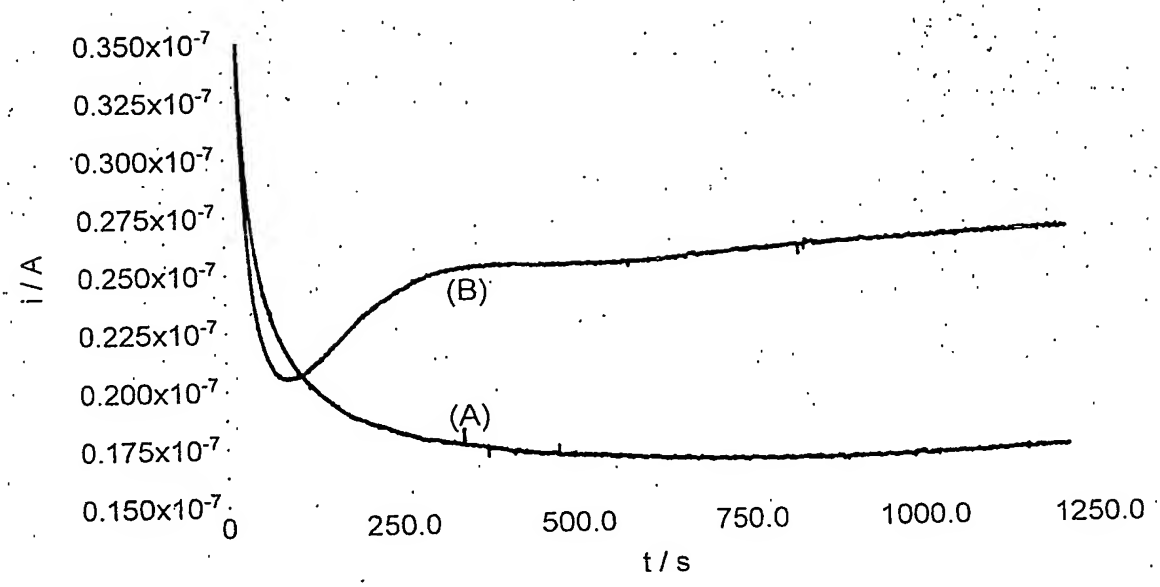
0.300x10-6
0.250x10-6
0.200x10-6
0.150x10-6
0.100x10-6
0.050x10-6
0
-0.050x10-6
i / A
0.250
0.300
0.350
0.400
0.450
0.500
0.550
0.600
E / V
(i)
(ii)
(iii)
(iv)

Figure 16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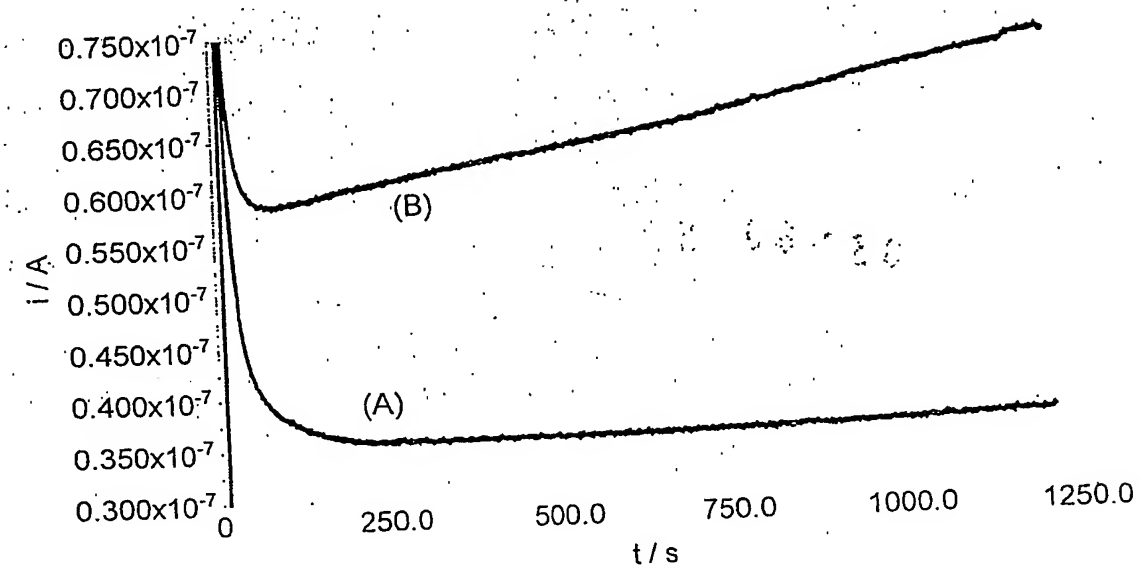
0.325x10-7
0.300x10-7
0.275x10-7
0.250x10-7
0.225x10-7
0.200x10-7
0.175x10-7
0.150x10-7
i / A
(B)
(A)
0
250.0
500.0
750.0
1000.0
1250.0
t / s

Figure 17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0.700x10-7
0.650x10-7
0.600x10-7
0.550x10-7
0.500x10-7
0.450x10-7
0.400x10-7
0.350x10-7
0.300x10-7
i / A
(B)
(A)
0
250.0
500.0
750.0
1000.0
1250.0
t / s

Figure 18